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ANTIBODIES TO HUMAN	) Our Ref.: TSRI 313.2 C1
IMMUNODEFICIENCY VIRUS	)
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HUMAN NEUTRALIZING MONOCLONAL ANTIBODIES
TO HUMAN IMMUNODEFICIENCY VIRUS

<u>Technical Field</u>

5   The present invention relates generally to the field of immunology and specifically to human monoclonal antibodies which bind and neutralize human immunodeficiency virus (HIV).

10   <u>Background</u>

1.   <u>HIV Immunotherapy</u>

HIV is the focus of intense studies as it is the causative agent for acquired immunodeficiency syndrome (AIDS). Immunotherapeutic methods are one of

15   several approaches to prevention, cure or remediation of HIV infection and HIV-induced diseases. Specifically, the use of neutralizing antibodies in passive immunotherapies is of central importance to the present invention.

20   Passive immunization of HIV-1 infected humans using human sera containing polyclonal antibodies immunoreactive with HIV has been reported. See for example, Jackson et al., <u>Lancet</u>, September 17:647-652, (1989); Karpas et al., <u>Proc. Natl. Acad. Sci., USA</u>,

25   87:7613-7616 (1990).

Numerous groups have reported the preparation of human monoclonal antibodies that neutralize HIV isolates <u>in vitro</u>. The described antibodies typically have immunospecificities for epitopes on the HIV

30   glycoprotein gp120 or the related external surface envelope glycoprotein gp120 or the transmembrane glycoprotein gp41. See, for example Levy, <u>Micro. Rev.</u>, 57:183-289 (1993); Karwowska et al., <u>Aids Research and Human Retroviruses</u>, 8:1099-1106 (1992);

35   Takeda et al., <u>J. Clin. Invest.</u>, 89:1952-1957 (1992);

Tilley et al., _Aids Research and Human Retroviruses_, 8:461-467 (1992); Laman et al., _J. Virol._, 66:1823-1831 (1992); Thali et al., _J. Virol._, 65:6188-6193 (1991); Ho et al., _Proc. Natl. Acad. Sci. USA_, 88:8949-8952 (1991); D'Souza et al., _AIDS_, 5:1061-1070 (1991); Tilley et al., _Res. Virol._, 142:247-259 (1991); Broliden et al., _Immunol._, 73:371-376 (1991); Matour et al., _J. Immunol._, 146:4325-4332 (1991); and Gorny et al., _Proc. Natl. Acad. Sci., USA_, 88:3238-3242 (1991).

To date, none of the reported human monoclonal antibodies have been shown to be effective in passive immunization therapies. Further, as monoclonal antibodies, they all each react with an individual epitope on the HIV envelope glycoprotein, gp120 or gp160. The epitope against which an effective neutralizing antibody immunoreacts has not been identified.

There continues to be a need to develop human monoclonal antibody preparations with significant HIV neutralization activity. In addition, there is a need for monoclonal antibodies immunoreactive with additional and diverse neutralizing epitopes on HIV gp120 and gp41 in view of recent studies suggesting that gp120 and gp41 are involved in both binding of the HIV virus to the cell as well as in post binding events including envelope shedding and cleavage. See, for review, Levy, _Micro. Rev._, 57:183-289 (1993). Additional (new) epitope specificities are required because, upon passive immunization, the administered patient can produce an immune response against the administered antibody, thereby inactivating the particular therapeutic antibody.

2.   Human Monoclonal Antibodies Produced From
      Combinatorial Phagemid Libraries

The use of filamentous phage display vectors,
referred to as phagemids, has been repeatedly shown to
allow the efficient preparation of large libraries of
monoclonal antibodies having diverse and novel
immunospecificities. The technology uses a
filamentous phage coat protein membrane anchor domain
as a means for linking gene-product and gene during
the assembly stage of filamentous phage replication,
and has been used for the cloning and expression of
antibodies from combinatorial libraries. Kang et al.,
Proc. Natl. Acad. Sci., USA, 88:4363-4366 (1991).
Combinatorial libraries of antibodies have been
produced using both the cpVIII membrane anchor (Kang
et al., supra) and the cpiii membrane anchor. Barbas
et al., Proc. Natl. Acad. Sci., USA, 88:7978-7982
(1991).

The diversity of a filamentous phage-based
combinatorial antibody library can be increased by
shuffling of the heavy and light chain genes (Kang et
al., Proc. Natl. Acad. Sci., USA, 88:11120-11123
(1991)), by altering the CDR3 regions of the cloned
heavy chain genes of the library (Barbas et al., Proc.
Natl. Acad. Sci., USA, 89:4457-4461 (1992)), and by
introducing random mutations into the library by
error-prone polymerase chain reactions (PCR) [Gram et
al., Proc. Natl. Acad. Sci., USA, 89:3576-3580
(1992)].

Filamentous phage display vectors have also been
utilized to produce human monoclonal antibodies
immunoreactive with hepatitis B virus (HBV) or HIV
antigens. See, for example Zebedee et al., Proc.
Natl. Acad. Sci., USA, 89:3175-3179 (1992); and Burton
et al., Proc. Natl. Acad. Sci., USA, 88:10134-10137

(1991), respectively. None of the previously described human monoclonal antibodies produced by phagemid vectors that are immunoreactive with HIV have been shown to neutralize HIV.

In particular, none of the previously-described human monoclonal antibodies produced by phagemid vectors are capable of neutralizing a majority of the field isolates of HIV. It is believed that certain of the antibodies described herein are particularly effective at neutralizing HIV because the antibodies immunoreact with an important antigenic determinant present on "mature" gp120 and not present on the HIV precursor protein gp160.

Brief Description of the Invention

Methods have now been discovered using the phagemid vectors to identify and isolate from combinatorial libraries human monoclonal antibodies that neutralize HIV, and allow the rapid preparation of large numbers of neutralizing antibodies of completely human derivation. The identified neutralizing antibodies define new epitopes on the HIV gp120 and gp41 glycoproteins, thereby increasing the availability of new immunotherapeutic human monoclonal antibodies.

The invention provides human monoclonal antibodies that neutralize HIV, and also provides cell lines used to produce these monoclonal antibodies.

Also provided are amino acid sequences which confer neutralization function to the antigen binding domain of a monoclonal antibody, and which can be used immunogenically to identify other antibodies that specifically bind and neutralize HIV. The monoclonal antibodies of the invention find particular utility as reagents for the diagnosis and immunotherapy of HIV-

induced disease.

A major advantage of the monoclonal antibodies of
the invention derives from the fact that they are
encoded by a human polynucleotide sequence. Thus, _in_
5   _vivo_ use of the monoclonal antibodies of the invention
for diagnosis and immunotherapy of HIV-induced disease
greatly reduces the problems of significant host
immune response to the passively administered
antibodies which is a problem commonly encountered
10   when monoclonal antibodies of xenogeneic or chimeric
derivation are utilized.

An additional major advantage of a preferred
group of monoclonal antibodies described herein
derives from the fact that they immunoreact with a
15   unique determinant present on mature HIV glycoprotein
gp120. This class of antibodies is particularly
effective at neutralizing field isolates of HIV.

In one embodiment, the invention contemplates a
human monoclonal antibody capable of immunoreacting
20   with human immunodeficiency virus (HIV) glycoprotein
gp120 and neutralizing HIV. A preferred human
monoclonal antibody has the binding specificity of a
monoclonal antibody comprising a heavy chain
immunoglobulin variable region amino acid residue
25   sequence selected from the group consisting of SEQ ID
Nos 66, 67, 68, 70, 72, 73, 74, 75, 78 and 97.

In a particularly preferred embodiment, the
invention describes a human monoclonal antibody
capable of immunoreacting with human immunodeficiency
30   virus (HIV) glycoprotein gp120 and neutralizing HIV,
wherein the monoclonal antibody has the capacity to
reduce HIV infectivity titer in an _in vitro_ virus
infectivity assay by 50% at a concentration of less
than 700 nanograms (ng) of antibody per milliliter
35   (ml).

Preferably, an anti-gp120 monoclonal antibody of this invention binds mature gp120 preferentially over HIV precursor glycoprotein gp160. More preferably, an anti-gp120 monoclonal antibody binds to a V1/V2 loop

5   deficient-variant gp120 substantially less than native gp120, thereby defining a important epitope for the antibody. Human monoclonal antibodies having these properties are particularly useful at neutralizing field isolates, and therefore provide useful

10 information regarding the immunocompetence of an immune response in HIV-infected patients.

Therefore, the invention provides for a screening method to determine whether HIV-infected patients contain antibodies of the class that neutralize field

15 isolates. The method for determining immunocompetence of a human anti-human immunodeficiency virus (HIV) antibody in a sample comprises the steps of:

(1)   contacting a sample believed to contain a human anti-HIV antibody with a diagnostically

20 effective amount of the above-described anti-gp120 monoclonal antibody in a competition immunoreaction admixture containing mature gp120 in the solid phase;

(2)   maintaining the competition immunoreaction admixture under conditions sufficient

25 for the monoclonal antibody to bind with the gp120 in the solid phase and form a solid phase immunoreactant; and

(3)   detecting the amount of the immunoreactant present in the solid phase, and thereby

30 the immunocompetence of any human anti-HIV antibody in the sample.

Another preferred human monoclonal antibody has the binding specificity of a monoclonal antibody comprising a light chain immunoglobulin variable

35 region amino acid residue sequence selected from the

group consisting of SEQ ID Nos 95, 96, 97, 98, 101, 102, 103, 104, 105, 107, 110, 115, 118, 121, 122, 124 and 132.

In a further embodiment, the invention contemplates a human monoclonal antibody capable of immunoreacting with human immunodeficiency virus (HIV) glycoprotein gp41 and neutralizing HIV. A preferred human monoclonal antibody has the binding specificity of a monoclonal antibody comprising a heavy chain immunoglobulin variable region amino acid residue sequence selected from the group consisting of SEQ ID Nos 142, 143, 144, 145 and 146. Another preferred human monoclonal antibody has the binding specificity of a monoclonal antibody comprising a light chain immunoglobulin variable region amino acid residue sequence selected from the group consisting of SEQ ID NOs 147, 148, 149, 150 and 151.

In another embodiment, the invention describes a polynucleotide sequence encoding a heavy or light chain immunoglobulin variable region amino acid residue sequence portion of a human monoclonal antibody of this invention. Also contemplated are DNA expression vectors containing the polynucleotide, and host cells containing the vectors and polynucleotides of the invention.

The invention also contemplates a method of detecting human immunodeficiency virus (HIV) comprising contacting a sample suspected of containing HIV with a diagnostically effective amount of the monoclonal antibody of this invention, and determining whether the monoclonal antibody immunoreacts with the sample. The method can be practiced _in vitro_ or _in vivo_, and may include a variety of methods for determining the presence of an immunoreaction product.

In another embodiment, the invention describes a

method for providing passive immunotherapy to human
immunodeficiency virus (HIV) disease in a human,
comprising administering to the human an
immunotherapeutically effective amount of the
monoclonal antibody of this invention. The
administration can be provided prophylactically, and
by a parenteral administration. Pharmaceutical
compositions containing one or more of the different
human monoclonal antibodies are described for use in
the therapeutic methods of the invention.

Brief Description of the Drawings

In the drawings forming a portion of this
disclosure:

Figure 1 illustrates the sequence of the
double-stranded synthetic DNA inserted into Lambda Zap
to produce a Lambda Hc2 expression vector. The
preparation of the double-stranded synthetic DNA
insert is described in Example 1a2). The various
features required for this vector to express the
V_H-coding DNA homologs include the Shine-Dalgarno
ribosome binding site, a leader sequence to direct the
expressed protein to the periplasm as described by
Mouva et al., J. Biol. Chem., 255:27, 1980, and
various restriction enzyme sites used to operatively
link the V_H homologs to the expression vector. The V_H
expression vector sequence also contains a short
nucleic acid sequence that codes for amino acids
typically found in variable regions heavy chain (V_H
backbone). This V_H backbone is just upstream and in
the proper reading as the V_H DNA homologs that are
operatively linked into the Xho I and Spe I cloning
sites. The sequences of the top and bottom strands of
the double-stranded synthetic DNA insert are listed
respectively in SEQ ID NO 1 and SEQ ID NO 2. The ten

amino acid sequence comprising the decapeptide tag is
listed in SEQ ID NO 5. The synthetic DNA insert is
directionally ligated into Lambda Zap II digested with
the restriction enzymes Not 1 and Xho I to form Lambda

5   Hc2 expression vector.

Figure 2 illustrates the major features of the
bacterial expression vector Lambda Hc2 (V_H expression
vector). The orientation of the insert in Lambda Zap
II is shown. The V_H DNA homologs are inserted into

10   the Xho I and Spe I cloning sites. The read through
transcription produces the decapeptide epitope (tag)
that is located just 3' of the cloning site. The
amino acid residue sequence of the decapeptide tag and
the Pel B leader sequence/spacer are respectively

15   listed in SEQ ID NO 5 and 6.

Figure 3 illustrates the sequence of the double-
stranded synthetic DNA inserted into Lambda Zap to
produce a Lambda Lc2 expression vector. The various
features required for this vector to express the V_L-

20   coding DNA homologs are described in Figure 1. The
V_L-coding DNA homologs are operatively linked into the
Lc2 sequence at the Sac I and Xho I restriction sites.
The sequences of the top and bottom strands of the
double-stranded synthetic DNA insert are listed

25   respectively in SEQ ID NO 3 and SEQ ID NO 4. The
synthetic DNA insert is directionally ligated into
Lambda Zap II digested with the restriction enzymes
Sac I and Not I to form Lambda Lc2 expression vector.

Figure 4 illustrates the major features of the

30   bacterial expression vector Lc2 (V_L expression
vector). The synthetic DNA sequence from Figure 3 is
shown at the top along with the LacZ promoter from
Lambda Zap II. The orientation of the insert in
Lambda Zap II is shown. The V_L DNA homologs are

35   inserted into the Sac I and Xho I cloning sites. The

amino acid residue sequence of the Pel B leader sequence/spacer is listed in SEQ ID NO 7.

Figure 5 illustrates the dicistronic expression vector, pComb, in the form of a phagemid expression vector.

Figure 6 illustrates the neutralization of HIV-1 by recombinant Fabs. The same supernate preparations were used in p24 and syncytia assays. The figures indicate neutralization titers. Refer to Example 3 for details of the assay procedures and discussion of the results. The ELISA titers and Fab concentrations were determined as described in Example 2b.

Figure 7 illustrates the relative affinities of Fab fragments for gp120 (IIIB) as illustrated by inhibition ELISA performed as described in Example 2b6). Fabs 27, 6, 29, 2 and 3 are all prototype members of the different groups discussed in Example 9a. Loop 2 is an Fab fragment selected from the same library as the other Fabs but which recognizes the V3 loop. The data is plotted as the percentage of maximum binding on the Y-axis against increasing concentrations (10^{-11} M to 10^{-7} M) of soluble gp120 on the X-axis.

Figure 8 illustrates the soluble CD4 competition with Fab fragments for gp120 (IIIB). P4D10 and loop2 are controls. P4D10 is a mouse monoclonal antibody reacting with the V3 loop of gp120 (IIIB). The data, discussed in Example 2b6), is plotted as described in Figure 7.

Figure 9 illustrates the neutralization of HIV by purified Fabs prepared as described in Example 3. The results shown are derived from the syncytia assay using the MN strain. The data is plotted as percent of inhibition of binding on the Y-axis against increasing Fab concentrations [0.1 to greater than 10

microgram/milliliter (μg/ml)] on the X-axis.

Figure 10 illustrates the amino acid residue
sequences of variable heavy (V_H) domains of Fabs
binding to gp120.   Seven distinct groups have been
identified as described in Example 9a based on
sequence homology.   Identity with the first sequence
in a group is indicated by dots.   The Fab clone names
are indicated in the left hand column.   The
corresponding SEQ ID Nos are indicated in the right
hand column.   The sequenced regions from right to left
are framework region 1 (FR1), complementary
determining region 1 (CDR1), framework region 2 (FR2),
complementary determining region 2 (CDR2), framework
region 3 (FR3), complementary determining region 3
(CDR3), and framework region 4 (FR4).   The five
amino-terminal residue sequence beginning with LEQ
arises from the VH1a while the 5 amino-terminal
residue sequence beginning with LEE arises from the
VH3a primers.   The b11 and b29 sequences are very
similar to the b3 group and could be argued to be
intraclonal variants within that group; they are
placed in their own group because of differences at
the V-D and D-J interface.

Figure 11 illustrates the amino acid residue
sequences of variable light (V_L) domains of Fabs
binding to gp120.   Refer to Figure 10 for the
description of the figure and to Example 9b for
analysis of the sequences.

Figure 12 illustrates the amino acid residue
sequences of V_L domains from Fabs binding to gp120 and
generated by shuffling the heavy chain from clone b12
against a library of light chains (H12-LCn Fabs) as
described in Example 10.   Note that the new V_L
sequences have designated clone numbers that do not
relate to those numbers from the original library.

The unique sequences are listed in the Sequence
Listing from SEQ ID NO 114 to 122. The new V_L domain
sequences are compared to that of the original clone
b12 V_L sequence.

5   Figure 13 illustrates the amino acid residue
sequences of V_H domains from Fabs binding to gp120 and
generated by shuffling the light chain from clone b12
against a library of heavy chains (L12-HCn Fabs) as
described in Example 10. Note that the new V_H
10   sequences have designated clone numbers that do not
relate to those numbers from the original library.
The unique sequences are listed in the Sequence
Listing from SEQ ID NO 123 to 132. The new V_H domain
sequences are compared to that of the original clone
15   b12 V_H sequence.

Figure 14 illustrates, in two figures, Figure 14A
and 14B, plasmid maps of the heavy (pTAC01H) and light
chain (pTC01) replicon-compatible chain-shuffling
vectors, respectively. Both plasmids are very similar
20   in the section containing the promoter and the cloning
site. Abbreviations: tacPO, tac promoter/operon; 5
histidine amino acid residue tag (histidine)5-tail;
f1IG, intergenic region of f1-phage; stu, stuffer
fragment ready for in-frame replacement by light and
25   heavy chain, respectively; cat, chloramphenicol
transferase gene; bla, b-lactamase gene; ori, origin
of replication. The map is drawn approximately to
scale.

Figure 15 illustrates the nucleotide sequences of
30   the binary shuffling vectors in two Figures, 15A and
15B. The construction and use of the vectors is
described in Example 11. In Figure 15A, the
double-stranded nucleotide sequence of the multiple
cloning site in light chain vector, pTC01, is shown.
35   The sequences of the top and bottom nucleotide base

strands are listed respectively in SEQ ID NO 8 and SEQ
ID NO 9. The amino acid residue sequence comprising
the pelB leader ending in the Sac I restriction site
is listed in SEQ ID NO 10. In Figure 15B, the

5   nucleotide sequence of the multiple cloning site in
heavy chain vector, pTAC01H, is shown. The sequences
of the top and bottom nucleotide base strands are
listed respectively in SEQ ID NO 11 and SEQ ID NO 12.
The amino acid residue sequence comprising the pelB

10   leader ending in the Xho I restriction site is listed
as SEQ ID NO 13. The amino acid residue sequence
comprising the histidine tail is listed in SEQ ID NO
14. Relevant restriction sites are underlined. tac
promoter and ribosome binding site (rbs) are indicated

15   by boxes.

Figure 16 illustrates the complete set of
directed crosses between heavy and light chains of all
Fab fragments isolated from the original library by
panning with gp160 (IIIB) (b1-b27), gp120 (IIIB)

20   (B8-B35), gp120 (SF2) (s4-s8), and the loop peptide
(p35) assayed by ELISA against IIIB gp120 as described
in Example 11. Heavy chains are listed horizontally
and light chains are listed vertically. Clones are
sorted according to the grouping established in

25   Example 9. Different groups are separated by
horizontal and vertical lines. A "-" at the
intersection of a particular heavy chain and light
chain signifies a clear negative (a signal of 3 times
background or less) for that particular cross, a "+"

30   shows a clear positive comparable to the original
heavy and light chain combination, and a "w" denotes
an intermediate value in the ELISA. "●": the HCp35/
LCp35 combination is negative when gp120 (IIIB) is
used, but positive when assayed with gp120 (IIIB).

35   Identical chains carry the same identifier (either *,

¶, §, or ¥).

Figure 17 illustrates the affinity of antibody-antigen interaction for b12 heavy chain crosses with light chains from all pannings analyzed

5   by competitive ELISA using soluble IIIB gp120 as competing antigen as described in Example 10. The data is plotted as the percentage of maximum binding on the Y-axis against increasing concentrations of soluble gp120 (IIIB) (10^{-12} M to 10^{-7} M) on the X-axis.

10 Figure 18 illustrates the amino acid residue sequences of variable heavy (V_H) domains of Fabs binding to gp41. The Fab clone names are indicated in the left hand column. The heavy chain sequences of the five Fabs individually designated DL 41 19, DO 41

15 11, GL 41 1, MT 41 12 and SS 41 8 have been assigned the respective SEQ ID Nos 142, 143, 144, 145 and 146. The sequenced regions from right to left are framework region 1 (FR1), complementary determining region 1 (CDR1), framework region 2 (FR2), complementary determining region 2 (CDR2), framework region 3 (FR3),

20 determining region 2 (CDR2), framework region 3 (FR3), complementary determining region 3 (CDR3), and framework region 4 (FR4).

Figure 19 illustrates the amino acid residue sequences of variable light (V_L) domains of Fabs

25 binding to gp41. Refer to Figure 18 for the description of the figure. The light chain sequences of the five Fabs individually designated DL 41 19, DO 41 11, GL 41 1, MT 41 12 and SS 41 8 have been assigned the respective SEQ ID NOs 147, 148, 149, 150

30 and 151.

Figure 20 illustrates the relative binding affinities of b3, b6, and b12 for the total envelope glycoproteins (gp160) and for the gp120 glycoprotein (gp120) expressed on the surface of COS-1 cells as

35 determined by immunoprecipitation and described in

Example 6. The signal on the autoradiogram represents
the relative amount of envelope glycoproteins bound
with increasing concentrations of Fab (0-150 µg/ml).

5   Figure 21 illustrates the neutralization of HIV-1
by b12 IgG1 as assessed using PHA-stimulated PBMCs as
indicator cells and determination of extracellular p24
as the reporter assay. Refer to Example 5d for
details of the assay procedures and discussion of the
results. The designation, location, and disease
10   status of the virus donors were as follows: ■, VS
(New York, acute), ▼, N70-2 (New Orleans,
asymptomatic), ▲, AC (San Diego, AIDS), ●, LS (Los
Angeles, AIDS), □, NYC-A (New York, unknown), ▽, WM
(Los Angeles, AIDS), △, RA (New York, acute), ◇, JP
15   (New York, acute). The molecularly cloned HIV-1 virus
JR-CSF (♦) and HIV-1 isolate JR-FL (o) were also
assayed for neutralization. The data is plotted as %
neutralization on the Y-axis against increasing
concentrations of b12 IgG1 (0-25 µg/ml) on the X-axis.
20   Figure 22 illustrates the reactivity of b12 IgG1
with a panel of international isolates of HIV-1 as
described in Example 8. Reactivity was determined
with gp120 isolated from the HIV-1 samples in ELISA
with the b12 IgG1 as described in Example 8. Data is
25   plotted as % b12 IgG1 reactivity on the X-axis against
clades A-F on the Y-axis. Country names indicate
where the HIV-1 virus was originally isolated. The
numbers in parenthesis refer to the number of viruses
of each clade examined. Reactivity is designated as
30   strong (▓) or moderate (∷).

Figure 23 illustrates the neutralization of the
HXBc2 molecular clone of HIV-1 LAI by purified Fabs
and a monoclonal antibody 110.4 (Mab 110.4) in an
envelope complementation assay as described in Example
35   3c. Neutralization of HXBc2 infectivity is expressed

as a decrease in residual CAT activity. The data is
plotted as % residual CAT activity on the Y-axis and
increasing concentrations of Fab and MAb (0.1-20
μg/ml) on the X-axis.

5   Figure 24 illustrates the pSG-5 mammalian
expression vector as described in Examples 4a and 4b.
Transcription of the heavy or light chain gene when
inserted in the EcoRI site is under the control of the
SV40 early promoter. Transcriptional termination is
10   signaled by the SV40 polyadenylation signal sequence
downstream of the heavy chain sequence. The M13
intergenic region allows for the production of single-
stranded DNA for nucleotide sequence determination.
The amp^R gene is for selection of the vector in
15   bacterial cells.

          Figures 25A and 25B illustrate the nucleotide and
amino acid residue sequences of the b12 light chain
gene in the pSG-5 mammalian expression vector
described in Example 4b. The b12 light chain has been
20   modified for expression in mammalian cells as
described in Example 4b.

          Figure 26 illustrates pEe6HC BM12, the pEE6
mammalian expression vector with the b12 IgG1 heavy
chain gene that has been modified for antibody
25   expression in mammalian cells as described in Example
4d. The VH was originally derived from the Fab b12
and has the same binding specificity as the Fab b12.
The pEE6 vector has a human CMV promoter for
expression of the heavy chain, a polyadenylation
30   signal for termination of transcription, and an
ampicillin gene for selection in bacteria.

          Figures 27A through 27E illustrate the nucleotide
and amino acid residue sequences of the b12 heavy
chain VH and constant regions in the pEe6HC BM12
35   mammalian expression vector as described Example 4d.

The b12 VH has been modified for expression in
mammalian cells as described in Example 4d.

Figure 28 illustrates pEe12 Combo BM12, the pEE12
mammalian expression vector with b12 IgG1 heavy and
light chain genes that have been modified for antibody
expression in mammalian cells as described in example
4f. The VH and light chain were originally derived
from the Fab b12 and have the same binding specificity
as the Fab b12. The pEE12 vector has a human CMV
promoter for expression of the light chain, a
polylinker to provide cloning sites, and a
polyadenylation signal for termination of
transcription. The vector also contains the GS
selectable marker gene whose expression is controlled
an SV40 early promoter at the 5' end of the GS gene,
an intron, and a polyadenylation signal at the 3' end
of the GS gene. A heavy chain cassette comprising the
HCMV promoter, enhancer elements, heavy chain gene,
and polyadenylation signal were removed from the pEE6
vector and inserted into the pEE12 vector to generate
the combinatorial construct containing both the b12
light and heavy chain genes.

Figure 29A through 29R illustrates the nucleotide
sequence of the pEE12 mammalian expression vector and
the b12 IgG1 heavy and light chain genes, pEe12 Combo
BM 12, as described in Example 4f. The VH and light
chain genes have been modified for expression in
mammalian cells as described in Example 4.

Detailed Description of the Invention

A.   Definitions

Amino Acid Residue: An amino acid formed
upon chemical digestion (hydrolysis) of a polypeptide
at its peptide linkages. The amino acid residues
described herein are preferably in the "L" isomeric

form. However, residues in the "D" isomeric form can
be substituted for any L-amino acid residue, as long
as the desired functional property is retained by the
polypeptide. NH_2 refers to the free amino group
present at the amino terminus of a polypeptide. COOH
refers to the free carboxy group present at the
carboxy terminus of a polypeptide. In keeping with
standard polypeptide nomenclature (described in J.
Biol. Chem., 243:3552-59 (1969) and adopted at 37 CFR
§1.822(b)(2)), abbreviations for amino acid residues
are shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

SYMBOL		AMINO ACID
1-Letter	3-Letter	
Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine
M	Met	methionine
A	Ala	alanine
S	Ser	serine
I	Ile	isoleucine
L	Leu	leucine
T	Thr	threonine
V	Val	valine
P	Pro	proline
K	Lys	lysine
H	His	histidine
Q	Gln	glutamine
E	Glu	glutamic acid
Z	Glx	Glu and/or Gln
W	Trp	tryptophan
R	Arg	arginine
D	Asp	aspartic acid
N	Asn	asparagine

B	Asx	Asn and/or Asp
C	Cys	cysteine
X	Xaa	Unknown or other

It should be noted that all amino acid residue
sequences represented herein by formulae have a left-
to-right orientation in the conventional direction of
amino terminus to carboxy terminus. In addition, the
phrase "amino acid residue" is broadly defined to
include the amino acids listed in the Table of
Correspondence and modified and unusual amino acids,
such as those listed in 37 CFR 1.822(b)(4), and
incorporated herein by reference. Furthermore, it
should be noted that a dash at the beginning or end of
an amino acid residue sequence indicates a peptide
bond to a further sequence of one or more amino acid
residues or a covalent bond to an amino-terminal group
such as NH_2 or acetyl or to a carboxy-terminal group
such as COOH.

Recombinant DNA (rDNA) molecule: A DNA molecule
produced by operatively linking two DNA segments.
Thus, a recombinant DNA molecule is a hybrid DNA
molecule comprising at least two nucleotide sequences
not normally found together in nature. RDNA'S not
having a common biological origin, i.e.,
evolutionarily different, are said to be
"heterologous".

Vector: A RDNA molecule capable of autonomous
replication in a cell and to which a DNA segment,
e.g., gene or polynucleotide, can be operatively
linked so as to bring about replication of the
attached segment. Vectors capable of directing the
expression of genes encoding for one or more
polypeptides are referred to herein as "expression
vectors". Particularly important vectors allow
cloning of cDNA (complementary DNA) from mRNAs

produced using reverse transcriptase.

Receptor: A receptor is a molecule, such as a
protein, glycoprotein and the like, that can
specifically (non-randomly) bind to another molecule.

5   Antibody: The term antibody in its various
grammatical forms is used herein to refer to
immunoglobulin molecules and immunologically active
portions of immunoglobulin molecules, i.e., molecules
that contain an antibody combining site or paratope.

10   Exemplary antibody molecules are intact immunoglobulin
molecules, substantially intact immunoglobulin
molecules and portions of an immunoglobulin molecule,
including those portions known in the art as Fab,
Fab', $F(ab')_2$ and $F(v)$.

15   Antibody Combining Site: An antibody combining
site is that structural portion of an antibody
molecule comprised of a heavy and light chain variable
and hypervariable regions that specifically binds
(immunoreacts with) an antigen. The term immunoreact

20   in its various forms means specific binding between an
antigenic determinant-containing molecule and a
molecule containing an antibody combining site such as
a whole antibody molecule or a portion thereof.

Monoclonal Antibody: A monoclonal antibody in

25   its various grammatical forms refers to a population
of antibody molecules that contain only one species of
antibody combining site capable of immunoreacting with
a particular epitope. A monoclonal antibody thus
typically displays a single binding affinity for any

30   epitope with which it immunoreacts. A monoclonal
antibody may therefore contain an antibody molecule
having a plurality of antibody combining sites, each
immunospecific for a different epitope, e.g., a
bispecific monoclonal antibody. Although historically

35   a monoclonal antibody was produced by immortalization

of a clonally pure immunoglobulin secreting cell line,
a monoclonally pure population of antibody molecules
can also be prepared by the methods of the present
invention.

5   Fusion Polypeptide: A polypeptide comprised of
at least two polypeptides and a linking sequence to
operatively link the two polypeptides into one
continuous polypeptide. The two polypeptides linked
in a fusion polypeptide are typically derived from two
10   independent sources, and therefore a fusion
polypeptide comprises two linked polypeptides not
normally found linked in nature.

Upstream: In the direction opposite to the
direction of DNA transcription, and therefore going
15   from 5' to 3' on the non-coding strand, or 3' to 5' on
the mRNA.

Downstream: Further along a DNA sequence in the
direction of sequence transcription or read out, that
is traveling in a 3'- to 5'-direction along the non-
20   coding strand of the DNA or 5'- to 3'-direction along
the RNA transcript.

Cistron: Sequence of nucleotides in a DNA
molecule coding for an amino acid residue sequence and
including upstream and downstream DNA expression
25   control elements.

Leader Polypeptide: A short length of amino acid
sequence at the amino end of a polypeptide, which
carries or directs the polypeptide through the inner
membrane and so ensures its eventual secretion into
30   the periplasmic space and perhaps beyond. The leader
sequence peptide is commonly removed before the
polypeptide becomes active.

Reading Frame: Particular sequence of contiguous
nucleotide triplets (codons) employed in translation.
35   The reading frame depends on the location of the

translation initiation codon.

B.   Human Monoclonal Antibodies

The present invention relates to human mono-
clonal antibodies which are specific for, and
neutralize human immunodeficiency virus (HIV). In a
preferred embodiment of the invention, human
monoclonal antibodies are disclosed which are capable
of binding epitopic polypeptide sequences in
glycoprotein gp120 of HIV. A further preferred
embodiment are human monoclonal antibodies capable of
binding epitopic polypeptide sequences in glycoprotein
gp 41 of HIV. Also disclosed is an antibody having a
specified amino acid sequence, which sequence confers
the ability to bind a specific epitope and to
neutralize HIV when the virus is bound by these
antibodies. A human monoclonal antibody with a
claimed specificity, and like human monoclonal
antibodies with like specificity, are useful in the
diagnosis and immunotherapy of HIV-induced disease.

The term "HIV-induced disease" means any disease
caused, directly or indirectly, by HIV. An example of
a HIV-induced disease is acquired autoimmunodeficiency
syndrome (AIDS), and any of the numerous conditions
associated generally with AIDS which are caused by HIV
infection.

Thus, in one aspect, the present invention is
directed to human monoclonal antibodies which are
reactive with a HIV neutralization site and cell lines
which produce such antibodies. The isolation of cell
lines producing monoclonal antibodies of the invention
is described in great detail further herein, and can
be accomplished using the phagemid vector library
methods described herein, and using routine screening
techniques which permit determination of the
elementary immunoreaction and neutralization patterns
of the monoclonal antibody of interest. Thus, if a
human monoclonal antibody being tested binds and
neutralizes HIV in a manner similar to a human
monoclonal antibody produced by the cell lines of the
invention then the tested antibody is considered
equivalent to an antibody of the invention.

It is also possible to determine, without undue
experimentation, if a human monoclonal antibody has
the same (i.e., equivalent) specificity as a human
monoclonal antibody of this invention by ascertaining
whether the former prevents the latter from binding to
HIV. If the human monoclonal antibody being tested
competes with the human monoclonal antibody of the
invention, as shown by a decrease in binding by the
human monoclonal antibody of the invention in standard
competition assays for binding to a solid phase
antigen, for example to gp120, then it is likely that
the two monoclonal antibodies bind to the same, or a
closely related, epitope.

Still another way to determine whether a human
monoclonal antibody has the specificity of a human
monoclonal antibody of the invention is to pre-
incubate the human monoclonal antibody of the
invention with HIV with which it is normally reactive,
and then add the human monoclonal antibody being

tested to determine if the human monoclonal antibody
being tested is inhibited in its ability to bind HIV.
If the human monoclonal antibody being tested is
inhibited then, in all likelihood, it has the same, or
5   functionally equivalent, epitopic specificity as the
monoclonal antibody of the invention. Screening of
human monoclonal antibodies of the invention, can be
also carried out utilizing HIV neutralization assays
and determining whether the monoclonal antibody
10   neutralizes HIV.

The ability to neutralize HIV at one or more
stages of virus infection is a desirable quality of a
human monoclonal antibody of the present invention.
Virus neutralization can be measured by a variety of
15   _in vitro_ and _in vivo_ methodologies. Exemplary methods
described herein for determining the capacity for
neutralization are the _in vitro_ assays that measure
inhibition of HIV-induced syncytia formation, plaque
assays and assays that measure the inhibition of
20   output of core p24 antigen from a cell infected with
HIV.

As shown herein, the immunospecificity of a human
monoclonal antibody of this invention can be directed
to epitopes that are shared across serotypes and/or
25   strains of HIV, or can be specific for a single strain
of HIV, depending upon the epitope. Thus, a preferred
human monoclonal antibody can immunoreact with HIV-1,
HIV-2, or both, and can immunoreact with one or more
of the HIV-1 strains IIIB, MN, RF, SF-2, Z2, Z6, CDC4,
30   ELI and the like strains. In addition, a preferred
human monoclonal antibody can immunoreact and
neutralize a majority of field isolates of HIV, as
described further herein.

The immunospecificity of an antibody, its HIV-
35   neutralizing capacity, and the attendant affinity the

antibody exhibits for the epitope, are defined by the
epitope with which the antibody immunoreacts.   The
epitope specificity is defined at least in part by the
amino acid residue sequence of the variable region of

5 the heavy chain of the immunoglobulin the antibody,
and in part by the light chain variable region amino
acid residue sequence.   Preferred human monoclonal
antibodies immunoreact with the CD4 binding site of
glycoprotein gp120.

10   Also disclosed is an antibody having a specified
amino acid sequence, which sequence confers the
ability to bind a specific unique neutralizing epitope
and to neutralize HIV when the virus is bound by these
antibodies.

15   A preferred human monoclonal antibody of this
invention has the binding specificity of a monoclonal
antibody comprising a heavy chain immunoglobulin
variable region amino acid residue sequence selected
from the group of sequences consisting of SEQ ID NOs

20 66, 67, 68, 70, 72, 73, 74, 75, 78 and 97, and
conservative substitutions thereof.

          Another preferred human monoclonal antibody of
this invention has the binding specificity of a
monoclonal antibody having a light chain

25 immunoglobulin variable region amino acid residue
sequence selected from the group of sequences
consisting of SEQ ID NOs 95, 96, 97, 98, 101, 102,
103, 104, 105, 107, 110, 115, 118, 121, 122, 124 and
132, and conservative substitutions thereof.

30   In a preferred embodiment, a monoclonal
antibodies of this invention exhibits a potent
capacity to neutralize HIV.   The capacity to
neutralize HIV is expressed as a concentration of
antibody molecules required to reduce the infectivity

35 titer of a suspension of HIV when assayed in an

typical _in vitro_ infectivity assay, such as is
described herein. A monoclonal antibody of this
invention has the capacity to reduce HIV infectivity
titer in an _in vitro_ virus infectivity assay by 50% at

5   a concentration of less than 700 nanograms (ng) of
antibody per milliliter (ml) of culture medium in the
assay, and preferably reduces infectivity titers 50%
at a concentration of less than 300 ng/ml, and more
preferably at concentrations less than about 10 ng/ml.

10   Exemplary and preferred monoclonal antibodies
described herein are effective at 3-700 ng/ml, and
therefore are particularly well suited for inhibiting
HIV _in vitro_ and _in vivo_.

Particularly preferred human monoclonal

15   antibodies of this invention immunoreact with gp120 in
its "mature" form, which form is to be distinguished
from antigenic determinants present on the HIV
envelope precursor glycoprotein designated gp160.
gp160 is processed during virus biogenesis by cleavage

20   into two polypeptides, gp41 and gp120. "Mature" gp120
refers to the processed protein that is found in
mature HIV virus particles, and can be detected on the
surface of HIV-infected cells.

Thus, a preferred antibody of this invention

25   binds mature gp120 preferentially over HIV precursor
glycoprotein gp160. By "binds preferentially" is
meant that the antibody immunoreacts with (binds)
substantially more mature gp120 than gp160 in an
immunoreaction admixture. Substantially more

30   typically indicates that at least greater than 50 % of
the total mass of immunoprecipitated material is
gp120, and preferably indicates that at least greater
than 75 %, more preferably 90 %, of the
immunoprecipitated material is gp120.

35   Methods for determining immunoreaction of a

subject antibody with gp120 or gp160 are well known in the art, and the invention need not be so limited. However, preferred methods for determining the relative amounts of envelope glycoprotein antigens are described in the Examples, and include radio-immunoprecipitation (RIP) of cell-surface labeled HIV-infected cells, followed by molecular weight analysis of the labeled products by polyacrylamide gel electrophoresis (PAGE).

A preferred human monoclonal antibody also has the ability to immunoreact with native gp120 and comparatively bind substantially less of a variant gp120 produced by recombinant DNA methods in which the V1 and V2 loops have been deleted. The variant gp120, also referred to a V1/V2 loop deficient-variant gp120, is described in the Examples, and is seen to bind substantially less of a preferred antibody, b12, in comparison to native gp120. The term "native gp120" refers to a mature gp120 protein having a normal amino acid residue sequence instead of a variant protein having selected amino acid residue substitutions or deletions, such as the V1/V2 loop deficient-variant in which the V1 and V2 loops were deleted. This preferential binding with native gp120 compared to the V1/V2 loop deficient-variant identifies an important epitope defined by a preferred antibody of this invention. Antibodies having this binding epitope are particularly effective at neutralizing a majority of field isolates of HIV, as described herein.

The ability to bind "substantially less" V1/V2 loop deficient-variant gp120 than native gp120 can be readily measured using various immunoreaction detection methods, although the assay methods described in Example 5c are particularly preferred. In preferred embodiments, substantially less binding

to V1/V2 loop deficient-variant gp120 compared to
native gp120 is indicated when the comparison is
conducted as described as in Example 5c, and the
native gp120 exhibits a ratio value deviating from the
5   mean of greater than 2.0 and the variant exhibits a
ratio value deviating from the mean of less than 0.5.

A particularly preferred human monoclonal
antibody of this invention also has the capacity to
neutralize a majority of field isolates as disclosed
10   herein. As is well understood, the field (i.e.,
clinically isolated) strains of HIV are typically
different to some degree antigenically from laboratory
strains. Therefor, it is well understood that useful
neutralizing antibodies must immunoreact with, and be
15   neutralizing against, field isolates of HIV.
Preferably, the useful antibody neutralized a large
percentage of field isolates, thereby increasing its
effectiveness when new strains are encountered.

The Examples demonstrate that the human
20   monoclonal antibody b12 has the ability to neutralize
a majority of the field isolates tested. By majority
is meant that in a representative and diverse
collection of field isolates, the antibody is capable
of neutralizing at least 50 % of the strains, and
25   preferably at least 75 % of the strains tested. In
this context, "neutralizing" means an effect of
reducing the HIV infectivity titre in an <u>in vitro</u>
virus infectivity assay as described herein at the
antibody concentrations described.
30   Thus, the invention also contemplates a human
monoclonal antibody capable of immunoreacting with and
neutralizing a first preselected human
immunodeficiency virus (HIV), such as the laboratory
isolate MN or IIIB, that is further capable of
35   immunoreacting with and neutralizing one or more other

(i.e., second) strains of HIV, particularly field strains. In this embodiment, supported by the teachings of the Examples, the antibody has the capacity to reduce HIV infectivity titer in an _in vitro_ virus infectivity assay of the first HIV strain by 50% at a concentration of at least less than 700 nanograms (ng) of antibody per milliliter (ml), and has the capacity to reduce HIV infectivity titer of a second field strain of HIV in the same _in vitro_ virus infectivity assay by 50% at a concentration of less than about 700 nanograms (ng) of antibody per milliliter (ml). In more preferred embodiments and depending upon the particular HIV strain, the capacity to reduce second field strain infectivity titers by 50% can be exhibited at lower antibody concentrations, such as below 300 ng/ml.

A particularly preferred antibody is an antibody having the binding specificity of the b12 monoclonal antibody described herein. The amino acid residue sequence of the heavy chain variable region of b12 is shown in SEQ ID NO 66, and the light chain variable region sequence of b12 is shown in SEQ ID NO 97. Still more preferred are human antibodies having the binding specificity of the immunoglobulin heavy and light chain polypeptides produced by ATCC 69079.

Further preferred human monoclonal antibodies immunoreact with the CD4 binding site of glycoprotein gp41. A preferred human monoclonal antibody of this invention has the binding specificity of a monoclonal antibody comprising a heavy chain immunoglobulin variable region amino acid residue sequence selected from the group of sequences consisting of SEQ ID NOs 142, 143, 144, 145, and 146 and conservative substitutions thereof.

Another preferred human monoclonal antibody of

this invention has the gp41 binding specificity of a
monoclonal antibody having a light chain
immunoglobulin variable region amino acid residue
sequence selected from the group of sequences

5   consisting of SEQ ID NOs 147, 148, 149, 150, and 151
and conservative substitutions thereof.

As shown by the present teachings and using the
combinatorial library shuffling and screening methods,
one can identify new heavy and light chain pairs (H:L)

10   that function as a HIV-neutralizing monoclonal
antibody. In particular, one can shuffle a known
heavy chain, derived from an HIV-neutralizing human
monoclonal antibody, with a library of light chains to
identify new H:L pairs that form a functional antibody

15   according to the present invention. Similarly, one
can shuffle a known light chain, derived from an HIV-
neutralizing human monoclonal antibody, with a library
of heavy chains to identify new H:L pairs that form a
functional antibody according to the present

20   invention.

Particularly preferred human monoclonal
antibodies are those having the gp120 immunoreaction
(binding) specificity of a monoclonal antibody having
heavy and light chain immunoglobulin variable region

25   amino acid residue sequences in pairs (H:L) selected
from the group consisting of SEQ ID NOs 66:95, 67:96,
72:102, 66:97, 73:107, 74:103, 70:101, 68:98, 75:104,
72:105, 78:110, 66:118, 66:122, 66:121, 66:115,
97:124, 97:132 and 66:98, and conservative

30   substitutions thereof. The designation of two SEQ ID
NOs with a colon, e.g., 66:95, is to connote a H:L
pair formed by the heavy and light chain,
respectively, amino acid residue sequences shown in
SEQ ID NO 66 and SEQ ID NO 95, respectively.

35   Further preferred human monoclonal antibodies are

those having the gp41 immunoreaction (binding)
specificity of a monoclonal antibody having heavy and
light chain immunoglobulin variable region amino acid
residue sequences in pairs (H:L) selected from the
group consisting of SEQ ID NOs 142:147, 143:148,
144:149, 145:150, and 146:151, and conservative
substitutions thereof.

Particularly preferred are human monoclonal
antibodies having the binding specificity of the
monoclonal antibody produced by the E. coli
microorganisms deposited with the ATCC, as described
further herein.

Particularly preferred are human monoclonal
antibodies having the binding specificity of the
monoclonal antibodies produced by the E. coli
microorganisms designated ATCC 69078, 69079 and 69080.
By "having the binding specificity" is meant
equivalent monoclonal antibodies which exhibit the
same or similar immunoreaction and neutralization
properties, and which compete for binding to an HIV
antigen. Preferred are the human monoclonal
antibodies produced by ATCC 69078, 69079 and 69080.

The term "conservative variation" as used herein
denotes the replacement of an amino acid residue by
another, biologically similar residue. Examples of
conservative variations include the substitution of
one hydrophobic residue such as isoleucine, valine,
leucine or methionine for another, or the substitution
of one polar residue for another, such as the
substitution of arginine for lysine, glutamic for
aspartic acids, or glutamine for asparagine, and the
like. The term "conservative variation" also includes
the use of a substituted amino acid in place of an
unsubstituted parent amino acid provided that
antibodies having the substituted polypeptide also

neutralize HIV. Analogously, another preferred
embodiment of the invention relates to polynucleotides
which encode the above noted heavy and/or light chain
polypeptides and to polynucleotide sequences which are
5   complementary to these polynucleotide sequences.
Complementary polynucleotide sequences include those
sequences which hybridize to the polynucleotide
sequences of the invention under stringent
hybridization conditions.

10   By using the human monoclonal antibodies of the
invention, it is now possible to produce anti-
idiotypic antibodies which can be used to screen human
monoclonal antibodies to identify whether the antibody
has the same binding specificity as a human monoclonal
15   antibody of the invention and also used for active
immunization (Herlyn et al., Science, 232:100 (1986)).
Such anti-idiotypic antibodies can be produced using
well-known hybridoma techniques (Kohler et al.,
Nature, 256:495 (1975)). An anti-idiotypic antibody
20   is an antibody which recognizes unique determinants
present on the human monoclonal antibody produced by
the cell line of interest. These determinants are
located in the hypervariable region of the antibody.
It is this region which binds to a given epitope and,
25   thus, is responsible for the specificity of the
antibody. An anti-idiotypic antibody can be prepared
by immunizing an animal with the monoclonal antibody
of interest. The immunized animal will recognize and
respond to the idiotypic determinants of the
30   immunizing antibody and produce an antibody to these
idiotypic determinants. By using the anti-idiotypic
antibodies of the immunized animal, which are specific
for the human monoclonal antibody of the invention
produced by a cell line which was used to immunize the
35   second animal, it is now possible to identify other

clones with the same idiotype as the antibody of the
hybridoma used for immunization. Idiotypic identity
between human monoclonal antibodies of two cell lines
demonstrates that the two monoclonal antibodies are
5   the same with respect to their recognition of the same
epitopic determinant. Thus, by using anti-idiotypic
antibodies, it is possible to identify other
hybridomas expressing monoclonal antibodies having the
same epitopic specificity.
10   It is also possible to use the anti-idiotype
technology to produce monoclonal antibodies which
mimic an epitope. For example, an anti-idiotypic
monoclonal antibody made to a first monoclonal
antibody will have a binding domain in the
15   hypervariable region which is the "image" of the
epitope bound by the first monoclonal antibody. Thus,
the anti-idiotypic monoclonal antibody can be used for
immunization, since the anti-idiotype monoclonal
antibody binding domain effectively acts as an
20   antigen.
           In one preferred embodiment, the invention
contemplates a truncated immunoglobulin molecule
comprising a Fab fragment derived from a human
monoclonal antibody of this invention. The Fab
25   fragment, lacking Fc receptor, is soluble, and affords
therapeutic advantages in serum half life, and
diagnostic advantages in modes of using the soluble
Fab fragment. The preparation of a soluble Fab
fragment is generally known in the immunological arts
30   and can be accomplished by a variety of methods. A
preferred method of producing a soluble Fab fragment
is described herein.
           In another preferred embodiment, the invention
contemplates an immunoglobulin molecule comprising a
35   Fab fragment derived from a human monoclonal antibody

of this invention and the fragment crystallizable (Fc) domain of a human immunoglobulin molecule. The entire (i.e., complete) immunoglobulin (Ig) molecule comprising a Fab fragment with the Fc domain may afford therapeutic and diagnostic advantages, and can be any of the several Ig species depending upon the ultimate use, including IgG, IgA, IgD, IgE, IgM, and isotypes thereof. The immunoglobulin molecule would be capable of effector functions associated with the Fc domain when used in passive immunotherapy. These effector functions include antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cellular cytotoxicity (CDCC) which promote the death of the cell to which the immunoglobulin molecule is specifically bound. The effector functions may therefore be desirable in therapeutic applications. Diagnostic assays include the ability to detect the presence of the immunoglobulin molecule. These assays rely on the cross-linking of red cells or beads in agglutinations, the activation of complement in plaque assays, or the antigenic properties of the Fc region of the heavy chain as detected by secondary antibodies in ELISA or RIA procedures to detect the presence of the immunoglobulin molecule. Such diagnostic assays can only be performed with the entire immunoglobulin molecule. The isolation of the immunoglobulin molecule is also facilitated by the presence of the Fc domain in that commonly used methods of immunoglobulin purification are based upon interaction of reagents with the Fc domain. The preparation of a Fab fragment with the Fc domain is generally known in the immunological arts and can be accomplished by a variety of methods. A preferred method of producing a Fab fragment with the Fc domain is described herein.

Particularly preferred is the immunoglobulin IgG1

human antibody described herein that is comprised of
the b12 antibody Fab fragment and human Fc domain
derived from an IgG1 subtype, designated b12 IgG1.
The structure and preparation of this preferred human

5   monoclonal antibody is described herein, and is
prepared using the recombinant DNA expression vector
pEE12. The complete nucleotide sequence of the vector
for expression the complete heavy and light chains in
the form of b12 IgG1 is shown in Figure 27 and also in

10 SEQ ID NO 156. Accordingly, the amino acid residue
and nucleotide sequences, respectively, for a
preferred complete heavy chain are shown in SEQ ID NOs
155 and 154, respectively, and for a preferred light
chain are shown in SEQ ID NOs 153, and 152,

15 respectively.

        C.   Immunotherapeutic Methods and Compositions
            The human monoclonal antibodies can also be
used immunotherapeutically for HIV disease. The term
"immunotherapeutically" or "immunotherapy" as used

20 herein in conjunction with the monoclonal antibodies
of the invention denotes both prophylactic as well as
therapeutic administration. Thus, the monoclonal
antibodies can be administered to high-risk patients

25 in order to lessen the likelihood and/or severity of
HIV-induced disease, administered to patients already
evidencing active HIV infection, or administered to
patients at risk of HIV infection.

30   1.   Therapeutic Compositions
            The present invention therefore
contemplates therapeutic compositions useful for
practicing the therapeutic methods described herein.
Therapeutic compositions of the present invention

35 contain a physiologically tolerable carrier together

with at least one species of human monoclonal antibody as described herein, dissolved or dispersed therein as an active ingredient. In a preferred embodiment, the therapeutic composition is not immunogenic when administered to a human patient for therapeutic purposes, unless that purpose is to induce an immune response, as described elsewhere herein.

As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a human without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like.

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art. Typically such compositions are prepared as sterile injectables either as liquid solutions or suspensions, aqueous or non-aqueous, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified.

The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the

effectiveness of the active ingredient.

The therapeutic composition of the present
invention can include pharmaceutically acceptable
salts of the components therein. Pharmaceutically
acceptable salts include the acid addition salts
(formed with the free amino groups of the polypeptide)
that are formed with inorganic acids such as, for
example, hydrochloric or phosphoric acids, or such
organic acids as acetic, tartaric, mandelic and the
like. Salts formed with the free carboxyl groups can
also be derived from inorganic bases such as, for
example, sodium, potassium, ammonium, calcium or
ferric hydroxides, and such organic bases as
isopropylamine, trimethylamine, 2-ethylamino ethanol,
histidine, procaine and the like.

Physiologically tolerable carriers are well known
in the art. Exemplary of liquid carriers are sterile
aqueous solutions that contain no materials in
addition to the active ingredients and water, or
contain a buffer such as sodium phosphate at
physiological pH value, physiological saline or both,
such as phosphate-buffered saline. Still further,
aqueous carriers can contain more than one buffer
salt, as well as salts such as sodium and potassium
chlorides, dextrose, propylene glycol, polyethylene
glycol and other solutes.

Liquid compositions can also contain liquid
phases in addition to and to the exclusion of water.
Exemplary of such additional liquid phases are
glycerin, vegetable oils such as cottonseed oil,
organic esters such as ethyl oleate, and water-oil
emulsions.

A therapeutic composition contains an HIV-
neutralizing of a human monoclonal antibody of the
present invention, typically an amount of at least 0.1

weight percent of antibody per weight of total
therapeutic composition. A weight percent is a ratio
by weight of antibody to total composition. Thus, for
example, 0.1 weight percent is 0.1 grams of antibody
5   per 100 grams of total composition.

2.   Therapeutic Methods

In view of the demonstrated HIV
neutralizing ability of the human monoclonal
10   antibodies of the present invention, the present
disclosure provides for a method for neutralizing HIV
in vitro or in vivo. The method comprises contacting
a sample believed to contain HIV with a composition
comprising a therapeutically effective amount of a
15   human monoclonal antibody of this invention.

For in vivo modalities, the method comprises
administering to the patient a therapeutically
effective amount of a physiologically tolerable
composition containing a human monoclonal antibody of
20   the invention. Thus, the present invention describes
in one embodiment a method for providing passive
immunotherapy to HIV disease in a human comprising
administering to the human an immunotherapeutically
effective amount of the monoclonal antibody of this
25   invention.

A representative patient for practicing the
present passive immunotherapeutic methods is any human
exhibiting symptoms of HIV-induced disease, including
AIDS or related conditions believed to be caused by
30   HIV infection, and humans at risk of HIV infection.
Patients at risk of infection by HIV include babies of
HIV-infected pregnant mothers, recipients of
transfusions known to contain HIV, users of HIV
contaminated needles, individuals who have
35   participated in high risk sexual activities with known

HIV-infected individuals, and the like risk
situations.

In one embodiment, the passive immunization
method comprises administering a composition
comprising more than one species of human monoclonal
antibody of this invention, preferably directed to
non-competing epitopes or directed to distinct
serotypes or strains of HIV, as to afford increased
effectiveness of the passive immunotherapy.

A therapeutically (immunotherapeutically)
effective amount of a human monoclonal antibody is a
predetermined amount calculated to achieve the desired
effect, i.e., to neutralize the HIV present in the
sample or in the patient, and thereby decrease the
amount of detectable HIV in the sample or patient. In
the case of in vivo therapies, an effective amount can
be measured by improvements in one or more symptoms
associated with HIV-induced disease occurring in the
patient, or by serological decreases in HIV antigens.

Thus, the dosage ranges for the administration of
the monoclonal antibodies of the invention are those
large enough to produce the desired effect in which
the symptoms of the HIV disease are ameliorated or the
likelihood of infection decreased. The dosage should
not be so large as to cause adverse side effects, such
as hyperviscosity syndromes, pulmonary edema,
congestive heart failure, and the like. Generally,
the dosage will vary with the age, condition, sex and
extent of the disease in the patient and can be
determined by one of skill in the art.

The dosage can be adjusted by the individual
physician in the event of any complication.

A therapeutically effective amount of an antibody
of this invention is typically an amount of antibody
such that when administered in a physiologically

tolerable composition is sufficient to achieve a
plasma concentration of from about 0.1 microgram (ug)
per milliliter (ml) to about 100 ug/ml, preferably
from about 1 ug/ml to about 5 ug/ml, and usually about

5   5 ug/ml. Stated differently, the dosage can vary from
about 0.1 mg/kg to about 300 mg/kg, preferably from
about 0.2 mg/kg to about 200 mg/kg, most preferably
from about 0.5 mg/kg to about 20 mg/kg, in one or more
dose administrations daily, for one or several days.

10   The human monoclonal antibodies of the invention
can be administered parenterally by injection or by
gradual infusion over time. Although the HIV
infection is typically systemic and therefore most
often treated by intravenous administration of

15   therapeutic compositions, other tissues and delivery
means are contemplated where there is a likelihood
that the tissue targeted contains infectious HIV.
Thus, human monoclonal antibodies of the invention can
be administered intravenously, intraperitoneally,

20   intramuscularly, subcutaneously, intracavity,
transdermally, and can be delivered by peristaltic
means.

          The therapeutic compositions containing a human
monoclonal antibody of this invention are

25   conventionally administered intravenously, as by
injection of a unit dose, for example. The term "unit
dose" when used in reference to a therapeutic
composition of the present invention refers to
physically discrete units suitable as unitary dosage

30   for the subject, each unit containing a predetermined
quantity of active material calculated to produce the
desired therapeutic effect in association with the
required diluent; i.e., carrier, or vehicle.

          The compositions are administered in a manner

35   compatible with the dosage formulation, and in a

therapeutically effective amount. The quantity to be
administered depends on the subject to be treated,
capacity of the subject's system to utilize the active
ingredient, and degree of therapeutic effect desired.
Precise amounts of active ingredient required to be
administered depend on the judgement of the
practitioner and are peculiar to each individual.
However, suitable dosage ranges for systemic
application are disclosed herein and depend on the
route of administration. Suitable regimes for
administration are also variable, but are typified by
an initial administration followed by repeated doses
at one or more hour intervals by a subsequent
injection or other administration. Alternatively,
continuous intravenous infusion sufficient to maintain
concentrations in the blood in the ranges specified
for _in vivo_ therapies are contemplated.

As an aid to the administration of effective
amounts of a monoclonal antibody, a diagnostic method
for detecting a monoclonal antibody in the subject's
blood is useful to characterize the fate of the
administered therapeutic composition.

The invention also relates to a method for
preparing a medicament or pharmaceutical composition
comprising the human monoclonal antibodies of the
invention, the medicament being used for immunotherapy
of HIV disease.

D.   Diagnostic Assay Methods

The present invention contemplates various
assay methods for determining the presence, and
preferably amount, of HIV in a sample such as a
biological fluid or tissue sample using a human
monoclonal antibody of this invention as an
immunochemical reagent to form an immunoreaction

product whose amount relates, either directly or indirectly, to the amount of HIV in the sample.

In a related embodiment, the present invention contemplates various assay methods for determining the presence, and preferably amount, of an anti-HIV antibody present in a sample such as a biological fluid or tissue sample from a HIV-infected individual using a human monoclonal antibody of this invention as an immunochemical reagent to form an immunoreaction product whose amount relates, either directly or indirectly, to the amount of anti-HIV antibody in the sample.

Those skilled in the art will understand that there are numerous well known clinical diagnostic chemistry procedures in which an immunochemical reagent of this invention can be used to form an immunoreaction product whose amount relates to the amount of HIV or anti-HIV antibody present in a body sample. Thus, while exemplary assay methods are described herein, the invention is not so limited.

Various heterogenous and homogeneous protocols, either competitive or noncompetitive, can be employed in performing an assay method of this invention. Examples of types of immunoassays which can utilize monoclonal antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA) and the sandwich (immunometric) assay.

Detection of the antigens using the monoclonal antibodies of the invention can be done utilizing immunoassays which are run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. Those of skill in the art will know, or can readily

discern, other immunoassay formats without undue experimentation.

The monoclonal antibodies of the invention can be bound to many different carriers and used to detect the presence of HIV. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding monoclonal antibodies, or will be able to ascertain such, using routine experimentation.

There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, and bio-luminescent compounds. Those of ordinary skill in the art will know of other suitable labels for binding to the monoclonal antibodies of the invention, or will be able to ascertain such, using routine experimentation. Furthermore, the binding of these labels to the monoclonal antibodies of the invention can be done using standard techniques common to those of ordinary skill in the art.

For purposes of the invention, HIV may be detected by the monoclonal antibodies of the invention when present in samples of biological fluids and tissues. Any sample containing a detectable amount of HIV can be used. A sample can be a liquid such as urine, saliva, cerebrospinal fluid, blood, serum and the like, or a solid or semi-solid such as tissues, feces, and the like, or, alternatively, a solid tissue

such as those commonly used in histological diagnosis.

Another labeling technique which may result in greater sensitivity consists of coupling the antibodies to low molecular weight haptens. These haptens can then be specifically detected by means of a second reaction. For example, it is common to use haptens such as biotin, which reacts with avidin, or dinitrophenol, pyridoxal, or fluorescein, which can react with specific anti-hapten antibodies.

The monoclonal antibodies of the invention are suited for use in vitro, for example, in immunoassays in which they can be utilized in liquid phase or bound to a solid phase carrier for the detection of HIV in samples, as described above. The monoclonal antibodies in these immunoassays can be detectably labeled in various ways for in vitro use.

In using the human monoclonal antibodies of the invention for the in vivo detection of antigen, the detectably labeled human monoclonal antibody is given in a dose which is diagnostically effective. The term "diagnostically effective" means that the amount of detectably labeled human monoclonal antibody is administered in sufficient quantity to enable detection of the site having the HIV antigen for which the monoclonal antibodies are specific.

The concentration of detectably labeled human monoclonal antibody which is administered should be sufficient such that the binding to HIV is detectable compared to the background. Further, it is desirable that the detectably labeled monoclonal antibody be rapidly cleared from the circulatory system in order to give the best target-to-background signal ratio.

As a rule, the dosage of detectably labeled human monoclonal antibody for in vivo diagnosis will vary depending on such factors as age, sex, and extent of

disease of the individual. The dosage of human monoclonal antibody can vary from about 0.01 mg/m^2 to about 500 mg/m^2, preferably 0.1 mg/m^2 to about 200 mg/m^2, most preferably about 0.1 mg/m^2 to about 10 mg/m^2. Such dosages may vary, for example, depending on whether multiple injections are given, tissue, and other factors known to those of skill in the art.

For _in vivo_ diagnostic imaging, the type of detection instrument available is a major factor in selecting a given radioisotope. The radioisotope chosen must have a type of decay which is detectable for a given type of instrument. Still another important factor in selecting a radioisotope for _in vivo_ diagnosis is that the half-life of the radioisotope be long enough so that it is still detectable at the time of maximum uptake by the target, but short enough so that deleterious radiation with respect to the host is minimized. Ideally, a radioisotope used for _in vivo_ imaging will lack a particle emission, but produce a large number of photons in the 140-250 keV range, which may be readily detected by conventional gamma cameras.

For _in vivo_ diagnosis radioisotopes may be bound to immunoglobulin either directly or indirectly by using an intermediate functional group. Intermediate functional groups which often are used to bind radioisotopes which exist as metallic ions to immunoglobulins are the bifunctional chelating agents such as diethylenetriaminepentacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA) and similar molecules. Typical examples of metallic ions which can be bound to the monoclonal antibodies of the invention are ^{111}In, ^{97}Ru, ^{67}Ga, ^{68}Ga, ^{72}As, ^{89}Zr, and ^{201}Tl.

The monoclonal antibodies of the invention can

also be labeled with a paramagnetic isotope for purposes of _in vivo_ diagnosis, as in magnetic resonance imaging (MRI) or electron spin resonance (ESR). In general, any conventional method for visualizing diagnostic imaging can be utilized. Usually gamma and positron emitting radioisotopes are used for camera imaging and paramagnetic isotopes for MRI. Elements which are particularly useful in such techniques include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Cr, and ^{56}Fe.

The human monoclonal antibodies of the invention can be used _in vitro_ and _in vivo_ to monitor the course of HIV disease therapy. Thus, for example, by measuring the increase or decrease in the number of cells infected with HIV or changes in the concentration of HIV present in the body or in various body fluids, it would be possible to determine whether a particular therapeutic regimen aimed at ameliorating the HIV disease is effective.

In a related diagnostic embodiment, the invention contemplates screening HIV-infected patients for the presence of circulating anti-HIV antibodies immunoreactive with gp120 that have a similar epitope immunospecificity when compared to a neutralizing antibody of this invention. Such a screening method indicates that the HIV-infected patient is exhibiting a significant immune response to the virus, and provides useful information regarding disease status and prognosis. The presence of anti-HIV antibodies cross-reactive with a neutralizing antibody of this invention indicates that the patient has some degree of HIV neutralizing activity, as defined herein.

The diagnostic assay involves determining whether the patient contains human anti-HIV antibodies immunoreactive with the same, similar or overlapping epitopes as a neutralizing antibody of the invention,

such that there is a likelihood that there is a useful
neutralizing immune response in the patient. There
are a variety of immunological assay formats that can
be utilized to determine cross-reactivity of test and
5   control antibodies, and the invention need not be so
limiting. Particularly preferred are competition
assays for a common antigen, preferably in the solid
phase.

10   A preferred embodiment of the competition
immunoassay method comprises the steps of:
(1)   contacting a sample believed to contain
a human anti-HIV antibody with a diagnostically
effective amount of the monoclonal antibody described
15   herein that binds mature gp120 in a competition
immunoreaction admixture containing mature gp120 in
the solid phase;
(2)   maintaining said competition
immunoreaction admixture under conditions sufficient
20   for said monoclonal antibody to bind with said gp120
in the solid phase and form a solid phase
immunoreactant; and
(3)   detecting the amount of said
immunoreactant present in said solid phase, and
25   thereby the immunocompetence of any human anti-HIV
antibody in said sample.
A diagnostically effective amount, in this
context, is a amount relative to the solid phase
gp120, preferably "mature" gp120 as defined herein,
30   sufficient to produce a detectable solid phase
immunoreaction product between the solid phase gp120
and the control anti-gp120 antibody of this invention.
Exemplary competition assays are described herein
using the preferred b12 antibody.
35   Conditions for conducting the competition

immunoreaction are well known in the art and can be
varied according to recognized parameters in the
contacting, the reaction admixtures, the maintenance
step, the immunoreaction conditions and the detecting
5   step. For example, the detection step can be
conducted by use of a labeled antibody of this
invention, by use of a second, labeled anti-human
antibody, and the like, as described herein.

10   E.   Diagnostic Systems
                   The present invention also describes a
diagnostic system, preferably in kit form, for
assaying for the presence of HIV or an anti-HIV
antibody in a sample according to the diagnostic
15   methods described herein. A diagnostic system
includes, in an amount sufficient to perform at least
one assay, a subject human monoclonal antibody, as a
separately packaged reagent.
              In another embodiment, a diagnostic system is
20   contemplated for assaying for the presence of an anti-
HIV monoclonal antibody in a body fluid sample such as
for monitoring the fate of therapeutically
administered antibody. The system includes, in an
amount sufficient for at least one assay, a subject
25   antibody as a control reagent, and preferably a
preselected amount of HIV antigen, each as separately
packaged immunochemical reagents.
              Instructions for use of the packaged reagent are
also typically included.
30   "Instructions for use" typically include a
tangible expression describing the reagent
concentration or at least one assay method parameter
such as the relative amounts of reagent and sample to
be admixed, maintenance time periods for reagent/
35   sample admixtures, temperature, buffer conditions and

the like.

    In embodiments for detecting HIV or anti-HIV antibody in a body fluid, a diagnostic system of the present invention can include a label or indicating means capable of signaling the formation of an immunocomplex containing a human monoclonal antibody of the present invention..

    The word "complex" as used herein refers to the product of a specific binding reaction such as an antibody-antigen reaction. Exemplary complexes are immunoreaction products.

    As used herein, the terms "label" and "indicating means" in their various grammatical forms refer to single atoms and molecules that are either directly or indirectly involved in the production of a detectable signal to indicate the presence of a complex. Any label or indicating means can be linked to or incorporated in an expressed protein, polypeptide, or antibody molecule that is part of an antibody or monoclonal antibody composition of the present invention, or used separately, and those atoms or molecules can be used alone or in conjunction with additional reagents. Such labels are themselves well-known in clinical diagnostic chemistry and constitute a part of this invention only insofar as they are utilized with otherwise novel proteins methods and/or systems.

    The labeling means can be a fluorescent labeling agent that chemically binds to antibodies or antigens without denaturing them to form a fluorochrome (dye) that is a useful immunofluorescent tracer. Suitable fluorescent labeling agents are fluorochromes such as fluorescein isocyanate (FIC), fluorescein isothiocyanate (FITC), 5-dimethylamine-1-naphthalenesulfonyl chloride (DANSC),

tetramethylrhodamine isothiocyanate (TRITC),
lissamine, rhodamine 8200 sulphonyl chloride (RB 200
SC) and the like. A description of immunofluorescence
analysis techniques is found in DeLuca,
"Immunofluorescence Analysis", in Antibody As a Tool,
Marchalonis et al., eds., John Wiley & Sons, Ltd., pp.
189-231 (1982), which is incorporated herein by
reference.

In preferred embodiments, the indicating group is
an enzyme, such as horseradish peroxidase (HRP),
glucose oxidase, or the like. In such cases where the
principal indicating group is an enzyme such as HRP or
glucose oxidase, additional reagents are required to
visualize the fact that a receptor-ligand complex
(immunoreactant) has formed. Such additional reagents
for HRP include hydrogen peroxide and an oxidation dye
precursor such as diaminobenzidine. An additional
reagent useful with glucose oxidase is 2,2'-amino-di-
(3-ethyl-benzthiazoline-G-sulfonic acid) (ABTS).

Radioactive elements are also useful labeling
agents and are used illustratively herein. An
exemplary radiolabeling agent is a radioactive element
that produces gamma ray emissions. Elements which
themselves emit gamma rays, such as ^{124}I, ^{125}I, ^{128}I, ^{132}I
and ^{51}Cr represent one class of gamma ray emission-
producing radioactive element indicating groups.
Particularly preferred is ^{125}I. Another group of
useful labeling means are those elements such as ^{11}C,
^{18}F, ^{15}O and ^{13}N which themselves emit positrons. The
positrons so emitted produce gamma rays upon
encounters with electrons present in the animal's
body. Also useful is a beta emitter, such ^{111} indium
of ^3H.

The linking of labels, i.e., labeling of,
polypeptides and proteins is well known in the art.

For instance, antibody molecules produced by a
hybridoma can be labeled by metabolic incorporation of
radioisotope-containing amino acids provided as a
component in the culture medium. See, for example,

5   Galfre et al., Meth. Enzymol., 73:3-46 (1981). The
techniques of protein conjugation or coupling through
activated functional groups are particularly
applicable. See, for example, Aurameas et al., Scand.
J. Immunol., Vol. 8 Suppl. 7:7-23 (1978), Rodwell et

10   al., Biotech., 3:889-894 (1984), and U.S. Pat. No.
4,493,795.

The diagnostic systems can also include,
preferably as a separate package, a specific binding
agent. A "specific binding agent" is a molecular

15   entity capable of selectively binding a reagent
species of the present invention or a complex
containing such a species, but is not itself a
polypeptide or antibody molecule composition of the
present invention. Exemplary specific binding agents

20   are second antibody molecules, complement proteins or
fragments thereof, S. aureus protein A, and the like.
Preferably the specific binding agent binds the
reagent species when that species is present as part
of a complex.

25   In preferred embodiments, the specific binding
agent is labeled. However, when the diagnostic system
includes a specific binding agent that is not labeled,
the agent is typically used as an amplifying means or
reagent. In these embodiments, the labeled specific

30   binding agent is capable of specifically binding the
amplifying means when the amplifying means is bound to
a reagent species-containing complex.

The diagnostic kits of the present invention can
be used in an "ELISA" format to detect the quantity of

35   an antigen or antibody of this invention in a vascular

fluid sample such as blood, serum, or plasma. "ELISA"
refers to an enzyme-linked immunosorbent assay that
employs an antibody or antigen bound to a solid phase
and an enzyme-antigen or enzyme-antibody conjugate to
detect and quantify the amount of an antigen present
in a sample. A description of the ELISA technique is
found in Chapter 22 of the 4th Edition of Basic and
Clinical Immunology by D.P. Sites et al., published by
Lange Medical Publications of Los Altos, CA in 1982
and in U.S. Patents No. 3,654,090; No. 3,850,752; and
No. 4,016,043, which are all incorporated herein by
reference.

Thus, in some embodiments, a human monoclonal
antibody of the present invention can be affixed to a
solid matrix to form a solid support that comprises a
package in the subject diagnostic systems.

A reagent is typically affixed to a solid matrix
by adsorption from an aqueous medium although other
modes of affixation applicable to proteins and
polypeptides well known to those skilled in the art,
can be used.

Useful solid matrices are also well known in the
art. Such materials are water insoluble and include
the cross-linked dextran available under the trademark
SEPHADEX from Pharmacia Fine Chemicals (Piscataway,
NJ); agarose; beads of polystyrene beads about 1
micron to about 5 millimeters in diameter available
from Abbott Laboratories of North Chicago, IL;
polyvinyl chloride, polystyrene, cross-linked
polyacrylamide, nitrocellulose- or nylon-based webs
such as sheets, strips or paddles; or tubes, plates or
the wells of a microtiter plate such as those made
from polystyrene or polyvinylchloride.

The reagent species, labeled specific binding
agent or amplifying reagent of any diagnostic system

described herein can be provided in solution, as a
liquid dispersion or as a substantially dry power,
e.g., in lyophilized form. Where the indicating means
is an enzyme, the enzyme's substrate can also be

5   provided in a separate package of a system. A solid
support such as the before-described microtiter plate
and one or more buffers can also be included as
separately packaged elements in this diagnostic assay
system.

10   The packaging materials discussed herein in
relation to diagnostic systems are those customarily
utilized in diagnostic systems.
           The term "package" refers to a solid matrix or
material such as glass, plastic (e.g., polyethylene,

15   polypropylene and polycarbonate), paper, foil and the
like capable of holding within fixed limits a
diagnostic reagent such as a monoclonal antibody of
the present invention. Thus, for example, a package
can be a bottle, vial, plastic and plastic-foil

20   laminated envelope or the like container used to
contain a contemplated diagnostic reagent or it can be
a microtiter plate well to which microgram quantities
of a contemplated diagnostic reagent have been
operatively affixed, i.e., linked so as to be capable

25   of being immunologically bound by an antibody or
polypeptide to be detected.
           The materials for use in the assay of the
invention are ideally suited for the preparation of a
kit. Such a kit may comprise a carrier means being

30   compartmentalized to receive in close confinement one
or more container means such as vials, tubes, and the
like, each of the container means comprising one of
the separate elements to be used in the method. For
example, one of the container means may comprise a

35   human monoclonal antibody of the invention which is,

or can be, detectably labelled. The kit may also have
containers containing any of the other above-recited
immunochemical reagents used to practice the
diagnostic methods.

5

        F.   Methods for Producing an HIV-Neutralizing
            Human Monoclonal Antibody

        The present invention describes methods for
producing novel HIV-neutralizing human monoclonal

10 antibodies. The methods are based generally on the
use of combinatorial libraries of antibody molecules
which can be produced from a variety of sources, and
include naive libraries, modified libraries, and
libraries produced directly from human donors

15 exhibiting an HIV-specific immune response.

        The combinatorial library production and
manipulation methods have been extensively described
in the literature, and will not be reviewed in detail
herein, except for those feature required to make and

20 use unique embodiments of the present invention.
However, the methods generally involve the use of a
filamentous phage (phagemid) surface expression vector
system for cloning and expressing antibody species of
the library. Various phagemid cloning systems to

25 produce combinatorial libraries have been described by
others. See, for example the preparation of
combinatorial antibody libraries on phagemids as
described by Kang et al., Proc. Natl. Acad. Sci., USA,
88:4363-4366 (1991); Barbas et al., Proc. Natl. Acad.

30 Sci., USA, 88:7978-7982 (1991); Zebedee et al., Proc.
Natl. Acad. Sci., USA, 89:3175-3179 (1992); Kang et
al., Proc. Natl. Acad. Sci., USA, 88:11120-11123
(1991); Barbas et al., Proc. Natl. Acad. Sci., USA,
89:4457-4461 (1992); and Gram et al., Proc. Natl.

35 Acad. Sci., USA, 89:3576-3580 (1992), which references

are hereby incorporated by reference.

    In one embodiment, the method involves preparing a phagemid library of human monoclonal antibodies by using donor immune cell messenger RNA from HIV-infected donors. The donors can be symptomatic of AIDS, but in preferred embodiments the donor is asymptomatic, as the resulting library contains a substantially higher number of HIV-neutralizing human monoclonal antibodies.

    In another embodiment, the donor is naive relative to an immune response to HIV, i.e., the donor is not HIV-infected. Alternatively, the library can be synthetic, or can be derived from a donor who has an immune response to other antigens.

    The method for producing a human monoclonal antibody generally involves (1) preparing separate H and L chain-encoding gene libraries in cloning vectors using human immunoglobulin genes as a source for the libraries, (2) combining the H and L chain encoding gene libraries into a single dicistronic expression vector capable of expressing and assembling a heterodimeric antibody molecule, (3) expressing the assembled heterodimeric antibody molecule on the surface of a filamentous phage particle, (4) isolating the surface-expressed phage particle using immunoaffinity techniques such as panning of phage particles against a preselected antigen, thereby isolating one or more species of phagemid containing particular H and L chain-encoding genes and antibody molecules that immunoreact with the preselected antigen.

    As described herein the Examples, the resulting phagemid library can be manipulated to increase and/or alter the immunospecificities of the monoclonal antibodies of the library to produce and subsequently

identify additional, desirable, human monoclonal antibodies of the present invention.

For example, the heavy (H) chain and light (L) chain immunoglobulin molecule encoding genes can be randomly mixed (shuffled) to create new HL pairs in an assembled immunoglobulin molecule. Additionally, either or both the H and L chain encoding genes can be mutagenized in the complementarity determining region (CDR) of the variable region of the immunoglobulin polypeptide, and subsequently screened for desirable immunoreaction and neutralization capabilities.

In one embodiment, the H and L genes can be cloned into separate, monocistronic expression vectors, referred to as a "binary" system described further herein. In this method, step (2) above differs in that the combining of H and L chain encoding genes occurs by the co-introduction of the two binary plasmids into a single host cell for expression and assembly of a phagemid having the surface accessible antibody heterodimer molecule.

In one shuffling embodiment, the shuffling can be accomplished with the binary expression vectors, each capable of expressing a single heavy or light chain encoding gene, as described in Example 11.

In the present methods, the antibody molecules are monoclonal because the cloning methods allow for the preparation of clonally pure species of antibody producing cell lines. In addition, the monoclonal antibodies are human because the H and L chain encoding genes are derived from human immunoglobulin producing immune cells, such as spleen, thymus, bone marrow, and the like.

The method of producing a HIV-neutralizing human monoclonal antibody also requires that the resulting antibody library, immunoreactive with a preselected

HIV antigen, is screened for the presence of antibody
species which have the capacity to neutralize HIV in
one or more of the assays described herein for
determining neutralization capacity. Thus, a
5   preferred library of antibody molecules is first
produced which binds to an HIV antigen, preferably
gp160, gp120, gp41, the V3 loop region of gp160, or
the CD4 binding site of gp120 and gp41, and then is
screened for the presence of HIV-neutralizing
10   antibodies as described herein.

Additional libraries can be screened from
shuffled libraries for additional HIV-immunoreactive
and neutralizing human monoclonal antibodies.

As a further characterization of the present
15   invention the nucleotide and corresponding amino acid
residue sequence of the antibody molecule's H or L
chain encoding gene is determined by nucleic acid
sequencing. The primary amino acid residue sequence
information provides essential information regarding
20   the antibody molecule's epitope reactivity.

Sequence comparisons of identified HIV-
immunoreactive monoclonal antibody variable chain
region sequences are shown herein in Figures 10-13.
The sequences are aligned based on sequence homology,
25   and groups of related antibody molecules are
identified thereby in which heavy chain or light chain
genes share substantial sequence homology.

An exemplary preparation of a human monoclonal
antibody is described in the Examples. The isolation
30   of a particular vector capable of expressing an
antibody of interest involves the introduction of the
dicistronic expression vector into a host cell
permissive for expression of filamentous phage genes
and the assembly of phage particles. Where the binary
35   vector system is used, both vectors are introduced in

the host cell. Typically, the host is E. coli. Thereafter, a helper phage genome is introduced into the host cell containing the immunoglobulin expression vector(s) to provide the genetic complementation necessary to allow phage particles to be assembled. The resulting host cell is cultured to allow the introduced phage genes and immunoglobulin genes to be expressed, and for phage particles to be assembled and shed from the host cell. The shed phage particles are then harvested (collected) from the host cell culture media and screened for desirable immunoreaction and neutralization properties. Typically, the harvested particles are "panned" for immunoreaction with a preselected antigen. The strongly immunoreactive particles are then collected, and individual species of particles are clonally isolated and further screened for HIV neutralization. Phage which produce neutralizing antibodies are selected and used as a source of a human HIV neutralizing monoclonal antibody of this invention.

Human monoclonal antibodies of this invention can also be produced by altering the nucleotide sequence of a polynucleotide sequence that encodes a heavy or light chain of a monoclonal antibody of this invention. For example, by site directed mutagenesis, one can alter the nucleotide sequence of an expression vector and thereby introduce changes in the resulting expressed amino acid residue sequence. Thus one can take the polynucleotide of SEQ ID NO 66, for example, and convert it into the polynucleotide of SEQ ID NO 67. Similarly, one can take a known polynucleotide and randomly alter it by random mutagenesis, reintroduce the altered polynucleotide into an expression system and subsequently screen the product H:L pair for HIV-neutralizing activity.

Site-directed and random mutagenesis methods are
well known in the polynucleotide arts, and are not to
be construed as limiting as methods for altering the
nucleotide sequence of a subject polynucleotide.

5   Due to the presence of the phage particle in an
immunoaffinity isolated antibody, one embodiment
involves the manipulation of the resulting cloned
genes to truncate the immunoglobulin-coding gene such
that a soluble Fab fragment is secreted by the host E.

10   coli cell containing the phagemid vector. Thus, the
resulting manipulated cloned immunoglobulin genes
produce a soluble Fab which can be readily
characterized in ELISA assays for epitope binding
studies, in competition assays with known anti-HIV

15   antibody molecules, and in HIV neutralization assays.
The solubilized Fab provides a reproducible and
comparable antibody preparation for comparative and
characterization studies.

The preparation of soluble Fab is generally
described in the immunological arts, and can be

20   conducted as described herein in Example 2b6), or as
described by Burton et al., Proc. Natl. Acad. Sci.,
USA, 88:10134-10137 (1991).

25   G.   Expression Vectors and Polynucleotides for
Expressing Anti-HIV Monoclonal Antibodies
The preparation of human monoclonal
antibodies of this invention depends, in one
embodiment, on the cloning and expression vectors used

30   to prepare the combinatorial antibody libraries
described herein. The cloned immunoglobulin heavy and
light chain genes can be shuttled between lambda
vectors, phagemid vectors and plasmid vectors at
various stages of the methods described herein.

35   The phagemid vectors produce fusion proteins that

are expressed on the surface of an assembled
filamentous phage particle.

A preferred phagemid vector of the present
invention is a recombinant DNA (rDNA) molecule
containing a nucleotide sequence that codes for and is
capable of expressing a fusion polypeptide containing,
in the direction of amino- to carboxy-terminus, (1) a
prokaryotic secretion signal domain, (2) a
heterologous polypeptide defining an immunoglobulin
heavy or light chain variable region, and (3) a
filamentous phage membrane anchor domain. The vector
includes DNA expression control sequences for
expressing the fusion polypeptide, preferably
prokaryotic control sequences.

The filamentous phage membrane anchor is
preferably a domain of the cpIII or cpVIII coat
protein capable of associating with the matrix of a
filamentous phage particle, thereby incorporating the
fusion polypeptide onto the phage surface.

The secretion signal is a leader peptide domain
of a protein that targets the protein to the
periplasmic membrane of gram negative bacteria. A
preferred secretion signal is a pelB secretion signal.
The predicted amino acid residue sequences of the
secretion signal domain from two pelB gene product
variants from Erwinia carotova are described in Lei et
al., Nature, 331:543-546 (1988).

The leader sequence of the pelB protein has
previously been used as a secretion signal for fusion
proteins (Better et al., Science, 240:1041-1043
(1988); Sastry et al., Proc. Natl. Acad. Sci., USA,
86:5728-5732 (1989); and Mullinax et al., Proc. Natl.
Acad. Sci., USA, 87:8095-8099 (1990)). Amino acid
residue sequences for other secretion signal
polypeptide domains from E. coli useful in this

invention as described in Oliver, <u>Escherichia coli</u>
and <u>Salmonella Typhimurium</u>, Neidhard, F.C. (ed.),
American Society for Microbiology, Washington, D.C.,
1:56-69 (1987).

Preferred membrane anchors for the vector are
obtainable from filamentous phage M13, f1, fd, and
equivalent filamentous phage. Preferred membrane
anchor domains are found in the coat proteins encoded
by gene III and gene VIII. The membrane anchor domain
of a filamentous phage coat protein is a portion of
the carboxy terminal region of the coat protein and
includes a region of hydrophobic amino acid residues
for spanning a lipid bilayer membrane, and a region of
charged amino acid residues normally found at the
cytoplasmic face of the membrane and extending away
from the membrane.

In the phage f1, gene VIII coat protein's
membrane spanning region comprises residue Trp-26
through Lys-40, and the cytoplasmic region comprises
the carboxy-terminal 11 residues from 41 to 52 (Ohkawa
et al., <u>J. Biol. Chem.</u>, 256:9951-9958 (1981)). An
exemplary membrane anchor would consist of residues 26
to 40 of cpVIII. Thus, the amino acid residue
sequence of a preferred membrane anchor domain is
derived from the M13 filamentous phage gene VIII coat
protein (also designated cpVIII or CP 8). Gene VIII
coat protein is present on a mature filamentous phage
over the majority of the phage particle with typically
about 2500 to 3000 copies of the coat protein.

In addition, the amino acid residue sequence of
another preferred membrane anchor domain is derived
from the M13 filamentous phage gene III coat protein
(also designated cpIII). Gene III coat protein is
present on a mature filamentous phage at one end of
the phage particle with typically about 4 to 6 copies

of the coat protein.

For detailed descriptions of the structure of filamentous phage particles, their coat proteins and particle assembly, see the reviews by Rached et al., _Microbiol. Rev._, 50:401-427 (1986); and Model et al., in "The Bacteriophages: Vol. 2", R. Calendar, ed. Plenum Publishing Co., pp. 375-456 (1988).

DNA expression control sequences comprise a set of DNA expression signals for expressing a structural gene product and include both 5' and 3' elements, as is well known, operatively linked to the cistron such that the cistron is able to express a structural gene product. The 5' control sequences define a promoter for initiating transcription and a ribosome binding site operatively linked at the 5' terminus of the upstream translatable DNA sequence.

To achieve high levels of gene expression in _E. coli_, it is necessary to use not only strong promoters to generate large quantities of mRNA, but also ribosome binding sites to ensure that the mRNA is efficiently translated. In _E. coli_, the ribosome binding site includes an initiation codon (AUG) and a sequence 3-9 nucleotides long located 3-11 nucleotides upstream from the initiation codon (Shine et al., _Nature_, 254:34 (1975). The sequence, AGGAGGU, which is called the Shine-Dalgarno (SD) sequence, is complementary to the 3' end of _E. coli_ 16S rRNA. Binding of the ribosome to mRNA and the sequence at the 3' end of the mRNA can be affected by several factors:

(i) The degree of complementarity between the SD sequence and 3' end of the 16S rRNA.

(ii) The spacing and possibly the DNA sequence lying between the SD sequence and the AUG. Roberts et al., _Proc. Natl. Acad. Sci., USA_, 76:760,

(1979a); Roberts et al., _Proc. Natl. Acad. Sci. USA_, 76:5596 (1979b); Guarente et al., _Science_, 209:1428 (1980); and Guarente et al., _Cell_, 20:543 (1980). Optimization is achieved by measuring the level of

5   expression of genes in plasmids in which this spacing is systematically altered. Comparison of different mRNAs shows that there are statistically preferred sequences from positions -20 to +13 (where the A of the AUG is position 0). Gold et al., _Annu. Rev._

10   _Microbiol._, 35:365 (1981). Leader sequences have been shown to influence translation dramatically. Roberts et al., 1979 a, b _supra_.

       (iii) The nucleotide sequence following the AUG, which affects ribosome binding. Taniguchi et

15   al., _J. Mol. Biol._, 118:533 (1978).

       The 3' control sequences define at least one termination (stop) codon in frame with and operatively linked to the heterologous fusion polypeptide.

       In preferred embodiments, the vector utilized

20   includes a prokaryotic origin of replication or replicon, i.e., a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extra chromosomally in a prokaryotic host cell, such as a bacterial host cell,

25   transformed therewith. Such origins of replication are well known in the art. Preferred origins of replication are those that are efficient in the host organism. A preferred host cell is _E. coli_. For use of a vector in _E. coli_, a preferred origin of

30   replication is ColE1 found in pBR322 and a variety of other common plasmids. Also preferred is the p15A origin of replication found on pACYC and its derivatives. The ColE1 and p15A replicon have been extensively utilized in molecular biology, are

35   available on a variety of plasmids and are described

at least by Sambrook et al., in "Molecular Cloning: a
Laboratory Manual", 2nd edition, Cold Spring Harbor
Laboratory Press (1989).

     The ColE1 and p15A replicons are particularly

5   preferred for use in one embodiment of the present
invention where two "binary" plasmids are utilized
because they each have the ability to direct the
replication of plasmid in E. coli while the other
replicon is present in a second plasmid in the same E.

10   coli cell. In other words, ColE1 and p15A are non-
interfering replicons that allow the maintenance of
two plasmids in the same host (see, for example,
Sambrook et al., supra, at pages 1.3-1.4). This
feature is particularly important in the binary

15   vectors embodiment of the present invention because a
single host cell permissive for phage replication must
support the independent and simultaneous replication
of two separate vectors, namely a first vector for
expressing a heavy chain polypeptide, and a second

20   vector for expressing a light chain polypeptide.

     In addition, those embodiments that include a
prokaryotic replicon can also include a gene whose
expression confers a selective advantage, such as drug
resistance, to a bacterial host transformed therewith.

25   Typical bacterial drug resistance genes are those that
confer resistance to ampicillin, tetracycline,
neomycin/kanamycin or cholamphenicol. Vectors
typically also contain convenient restriction sites
for insertion of translatable DNA sequences.

30   Exemplary vectors are the plasmids pUC8, pUC9, pBR322,
and pBR329 available from BioRad Laboratories,
(Richmond, CA) and pPL and pKK223 available from
Pharmacia, (Piscataway, NJ).

     A vector for expression of a monoclonal antibody

35   of the invention on the surface of a filamentous phage

particle is a recombinant DNA (rDNA) molecule adapted
for receiving and expressing translatable first and
second DNA sequences in the form of first and second
polypeptides wherein one of the polypeptides is fused
5   to a filamentous phage coat protein membrane anchor.
That is, at least one of the polypeptides is a fusion
polypeptide containing a filamentous phage membrane
anchor domain, a prokaryotic secretion signal domain,
and an immunoglobulin heavy or light chain variable
10 domain.

A DNA expression vector for expressing a
heterodimeric antibody molecule provides a system for
independently cloning (inserting) the two translatable
DNA sequences into two separate cassettes present in
15 the vector, to form two separate cistrons for
expressing the first and second polypeptides of the
antibody molecule, or the ligand binding portions of
the polypeptides that comprise the antibody molecule
(i.e., the H and L variable regions of an
20 immunoglobulin molecule). The DNA expression vector
for expressing two cistrons is referred to as a
dicistronic expression vector.

The vector comprises a first cassette that
includes upstream and downstream translatable DNA
25 sequences operatively linked via a sequence of
nucleotides adapted for directional ligation to an
insert DNA. The upstream translatable sequence
encodes the secretion signal as defined herein. The
downstream translatable sequence encodes the
30 filamentous phage membrane anchor as defined herein.
The cassette preferably includes DNA expression
control sequences for expressing the receptor
polypeptide that is produced when an insert
translatable DNA sequence (insert DNA) is
35 directionally inserted into the cassette via the

sequence of nucleotides adapted for directional
ligation. The filamentous phage membrane anchor is
preferably a domain of the cpIII or cpVIII coat
protein capable of binding the matrix of a filamentous
5   phage particle, thereby incorporating the fusion
polypeptide onto the phage surface.

The receptor expressing vector also contains a
second cassette for expressing a second receptor
polypeptide. The second cassette includes a second
10   translatable DNA sequence that encodes a secretion
signal, as defined herein, operatively linked at its
3' terminus via a sequence of nucleotides adapted for
directional ligation to a downstream DNA sequence of
the vector that typically defines at least one stop
15   codon in the reading frame of the cassette. The
second translatable DNA sequence is operatively linked
at its 5' terminus to DNA expression control sequences
forming the 5' elements. The second cassette is
capable, upon insertion of a translatable DNA sequence
20   (insert DNA), of expressing the second fusion
polypeptide comprising a receptor of the secretion
signal with a polypeptide coded by the insert DNA.

An upstream translatable DNA sequence encodes a
prokaryotic secretion signal as described earlier.
25   The upstream translatable DNA sequence encoding the
pelB secretion signal is a preferred DNA sequence for
inclusion in a receptor expression vector. A
downstream translatable DNA sequence encodes a
filamentous phage membrane anchor as described
30   earlier. Thus, a downstream translatable DNA sequence
encodes an amino acid residue sequence that
corresponds, and preferably is identical, to the
membrane anchor domain of either a filamentous phage
gene III or gene VIII coat polypeptide.
35   A cassette in a DNA expression vector of this

invention is the region of the vector that forms, upon insertion of a translatable DNA sequence (insert DNA), a sequence of nucleotides capable of expressing, in an appropriate host, a fusion polypeptide. The

5   expression-competent sequence of nucleotides is referred to as a cistron. Thus, the cassette comprises DNA expression control elements operatively linked to the upstream and downstream translatable DNA sequences. A cistron is formed when a translatable

10   DNA sequence is directionally inserted (directionally ligated) between the upstream and downstream sequences via the sequence of nucleotides adapted for that purpose. The resulting three translatable DNA sequences, namely the upstream, the inserted and the

15   downstream sequences, are all operatively linked in the same reading frame.

Thus, a DNA expression vector for expressing an antibody molecule provides a system for cloning translatable DNA sequences into the cassette portions

20   of the vector to produce cistrons capable of expressing the first and second polypeptides, i.e., the heavy and light chains of a monoclonal antibody.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting between different genetic environments another nucleic acid to

25   which it has been operatively linked. Preferred vectors are those capable of autonomous replication and expression of structural gene products present in the DNA segments to which they are operatively linked.

30   Vectors, therefore, preferably contain the replicons and selectable markers described earlier.

As used herein with regard to DNA sequences or segments, the phrase "operatively linked" means the sequences or segments have been covalently joined,

35   preferably by conventional phosphodiester bonds, into

one strand of DNA, whether in single or double
stranded form. The choice of vector to which
transcription unit or a cassette of this invention is
operatively linked depends directly, as is well known
5   in the art, on the functional properties desired,
e.g., vector replication and protein expression, and
the host cell to be transformed, these being
limitations inherent in the art of constructing
recombinant DNA molecules.

10   A sequence of nucleotides adapted for directional
ligation, i.e., a polylinker, is a region of the DNA
expression vector that (1) operatively links for
replication and transport the upstream and downstream
translatable DNA sequences and (2) provides a site or
15   means for directional ligation of a DNA sequence into
the vector. Typically, a directional polylinker is a
sequence of nucleotides that defines two or more
restriction endonuclease recognition sequences, or
restriction sites. Upon restriction cleavage, the two
20   sites yield cohesive termini to which a translatable
DNA sequence can be ligated to the DNA expression
vector. Preferably, the two restriction sites
provide, upon restriction cleavage, cohesive termini
that are non-complementary and thereby permit
25   directional insertion of a translatable DNA sequence
into the cassette. In one embodiment, the directional
ligation means is provided by nucleotides present in
the upstream translatable DNA sequence, downstream
translatable DNA sequence, or both. In another
30   embodiment, the sequence of nucleotides adapted for
directional ligation comprises a sequence of
nucleotides that defines multiple directional cloning
means. Where the sequence of nucleotides adapted for
directional ligation defines numerous restriction
35   sites, it is referred to as a multiple cloning site.

In a preferred embodiment, a DNA expression
vector is designed for convenient manipulation in the
form of a filamentous phage particle encapsulating a
genome according to the teachings of the present
invention. In this embodiment, a DNA expression
vector further contains a nucleotide sequence that
defines a filamentous phage origin of replication such
that the vector, upon presentation of the appropriate
genetic complementation, can replicate as a
filamentous phage in single stranded replicative form
and be packaged into filamentous phage particles.
This feature provides the ability of the DNA
expression vector to be packaged into phage particles
for subsequent segregation of the particle, and vector
contained therein, away from other particles that
comprise a population of phage particles.

A filamentous phage origin of replication is a
region of the phage genome, as is well known, that
defines sites for initiation of replication,
termination of replication and packaging of the
replicative form produced by replication (see, for
example, Rasched et al., <u>Microbiol. Rev.</u>, 50:401-427
(1986); and Horiuchi, <u>J. Mol. Biol.</u>, 188:215-223
(1986)).

A preferred filamentous phage origin of
replication for use in the present invention is an
M13, f1 or fd phage origin of replication (Short et
al., <u>Nucl. Acids Res.</u>, 16:7583-7600 (1988)).
Preferred DNA expression vectors for cloning and
expression a human monoclonal antibody of this
invention are the dicistronic expression vectors
pComb8, pComb2-8, pComb3, pComb2-3 and pComb2-3',
described herein.

A particularly preferred vector of the present
invention includes a polynucleotide sequence that

encodes a heavy or light chain variable region of a
human monoclonal antibody of the present invention.
Particularly preferred are vectors that include a
nucleotide sequence that encodes a heavy or light

5 chain amino acid residue sequence shown in Figures 10-
13, that encodes a heavy or light chain having the
binding specificity of those sequences shown in
Figures 10-13, or that encodes a heavy or light chain
having conservative substitutions relative to a

10 sequence shown in Figures 10-13, and complementary
polynucleotide sequences thereto.

Insofar as polynucleotides are component parts of
a DNA expression vector for producing a human
monoclonal antibody heavy or light chain

15 immunoglobulin variable region amino acid residue
sequence, the invention also contemplates isolated
polynucleotides that encode such heavy or light chain
sequences.

It is to be understood that, due to the genetic

20 code and its attendant redundancies, numerous
polynucleotide sequences can be designed that encode a
contemplated heavy or light chain immunoglobulin
variable region amino acid residue sequence. Thus,
the invention contemplates such alternate

25 polynucleotide sequences incorporating the features of
the redundancy of the genetic code.

Insofar as the expression vector for producing a
human monoclonal antibody of this invention is carried
in a host cell compatible with expression of the

30 antibody, the invention contemplates a host cell
containing a vector or polynucleotide of this
invention. A preferred host cell is E. coli, as
described herein.

E. coli cultures containing preferred expression

35 vectors that produce a human monoclonal antibody of

this invention were deposited pursuant to Budapest
Treaty requirements with the American Type Culture
Collection (ATCC), Rockville, MD, as described herein.

5   Examples

     The following examples are intended to
illustrate, but not limit, the scope of the invention.

1.   Construction of a Dicistronic Expression Vector
10   for Producing a Heterodimeric Receptor on Phage
          Particles
          To obtain a vector system for generating a large
number of Fab antibody fragments that can be screened
directly, expression libraries in bacteriophage Lambda
15   have previously been constructed as described in Huse
et al., Science, 246:1275-1281 (1989). These systems
did not contain design features that provide for the
expressed Fab to be targeted to the surface of a
filamentous phage particle.
20   The main criterion used in choosing a vector
system was the necessity of generating the largest
number of Fab fragments which could be screened
directly. Bacteriophage Lambda was selected as the
starting point to develop an expression vector for
25   three reasons. First, in vitro packaging of phage DNA
was the most efficient method of reintroducing DNA
into host cells. Second, it was possible to detect
protein expression at the level of single phage
plaques. Finally, the screening of phage libraries
30   typically involved less difficulty with nonspecific
binding. The alternative, plasmid cloning vectors,
are only advantageous in the analysis of clones after
they have been identified. This advantage was not
lost in the present system because of the use of a
35   dicistronic expression vector such as pCombVIII,

thereby permitting a plasmid containing the heavy
chain, light chain, or Fab expressing inserts to be
excised.

    a.   <u>Construction of Dicistronic Expression</u>

          <u>Vector pCOMB</u>

          1)   <u>Preparation of Lambda Zap ™II</u>

          Lambda Zap™ II is a derivative of the
original Lambda Zap (ATCC Accession No. 40,298) that
maintains all of the characteristics of the original
Lambda Zap including 6 unique cloning sites, fusion
protein expression. and the ability to rapidly excise
the insert in the form of a phagemid (Bluescript SK-),
but lacks the SAM 100 mutation, allowing growth on
many Non-Sup F strains, including XL1-Blue. The
Lambda Zap™ II was constructed as described in Short
et al., <u>Nuc. Acids Res.</u>, 16:7583-7600, 1988, by
replacing the Lambda S gene contained in a 4254 base
pair (bp) DNA fragment produced by digesting Lambda
Zap with the restriction enzyme Nco I. This 4254 bp
DNA fragment was replaced with the 4254 bp DNA
fragment containing the Lambda S gene isolated from
Lambda gt10 (ATCC # 40,179) after digesting the vector
with the restriction enzyme Nco I. The 4254 bp DNA
fragment isolated from lambda gt10 was ligated into
the original Lambda Zap vector using T4 DNA ligase and
standard protocols such as those described in <u>Current</u>
<u>Protocols in Molecular Biology</u>, Ausubel et al., eds.,
John Wiley and Sons, NY, 1987, to form Lambda Zap™
II.

          2)   <u>Preparation of Lambda Hc2</u>

          To express a plurality of V_H-coding DNA
homologs in an <u>E. coli</u> host cell, a vector designated
Lambda Hc2 was constructed. The vector provided the
following: the capacity to place the V_H-coding DNA
homologs in the proper reading frame; a ribosome

binding site as described by Shine et al., <u>Nature,</u>
254:34 (1975); a leader sequence directing the
expressed protein to the periplasmic space designated
the pelB secretion signal; a polynucleotide sequence
that coded for a known epitope (epitope tag); and also
a polynucleotide that coded for a spacer protein
between the V_H-coding DNA homolog and the
polynucleotide coding for the epitope tag. Lambda Hc2
has been previously described by Huse et al., <u>Science,</u>
246:1275-1281 (1989).

To prepare Lambda Hc2, a synthetic DNA sequence
containing all of the above features was constructed
by designing single stranded polynucleotide segments
of 20-40 bases that would hybridize to each other and
form the double stranded synthetic DNA sequence shown
in Figure 1. The individual single-stranded
polynucleotide segments are shown in Table 1.

Polynucleotides N2, N3, N9-4, N11, N10-5, N6, N7
and N8 (Table 1) were kinased by adding 1 μl of each
polynucleotide 0.1 micrograms/microliter (μg/μl) and
20 units of T_4 polynucleotide kinase to a solution
containing 70 mM Tris-HCl (Tris[hydroxymethyl]
aminomethane hydrochloride) at pH 7.6, 10 mM $MgCl_2$, 5
mM dithiothreitol (DTT), 10 mM beta-mercaptoethanol,
500 micrograms per milliliter (μg/ml) bovine serum
albumin (BSA). The solution was maintained at 37
degrees Centigrade (37°C) for 30 minutes and the
reaction stopped by maintaining the solution at 65°C
for 10 minutes. The two end polynucleotides, 20
nanograms (ng) of polynucleotides N1 and
polynucleotides N12, were added to the above kinasing
reaction solution together with 1/10 volume of a
solution containing 20 mM Tris-HCl at pH 7.4, 2.0 mM
$MgCl_2$ and 50 mM NaCl. This solution was heated to
70°C for 5 minutes and allowed to cool to room

temperature, approximately 25°C, over 1.5 hours in a
500 ml beaker of water. During this time period all
10 polynucleotides annealed to form the double
stranded synthetic DNA insert shown in Figure 1. The
5   individual polynucleotides were covalently linked to
each other to stabilize the synthetic DNA insert by
adding 40 μl of the above reaction to a solution
containing 50 mM Tris-HCl at pH 7.5, 7 mM MgCl$_2$, 1 mM
DTT, 1 mM adenosine triphosphate (ATP) and 10 units of
10 T4 DNA ligase. This solution was maintained at 37°C
for 30 minutes and then the T4 DNA ligase was
inactivated by maintaining the solution at 65°C for 10
minutes. The end polynucleotides were kinased by
mixing 52 μl of the above reaction, 4 μl of a solution
15 containing 10 mM ATP and 5 units of T4 polynucleotide
kinase. This solution was maintained at 37°C for 30
minutes and then the T4 polynucleotide kinase was
inactivated by maintaining the solution at 65°C for 10
minutes.

20

Table 1

SEQ
ID NO
(15)   N1)   5' GGCCGCAAATTCTATTTCAAGGAGACAGTCAT 3'
25 (16)   N2)   5' AATGAAATACCTATTGCCTACGGCAGCCGCTGGATT 3'
(17)   N3)   5' GTTATTACTCGCTGCCCAACCAGCCATGGCCC 3'
(18)   N6)   5' CAGTTTCACCTGGGCCATGGCTGGTTGGG 3'
(19)   N7)   5' CAGCGAGTAATAACAATCCAGCGGCTGCCGTAGGCAATAG 3'
(20)   N8)   5' GTATTTCATTATGACTGTCTCCTTGAAATAGAATTTGC 3'
30 (21)   N9-4) 5' AGGTGAAACTGCTCGAGATTTCTAGACTAGTTACCCGTAC 3'
(22)   N10-5) 5' CGGAACGTCGTACGGGTAACTAGTCTAGAAATCTCGAG 3'
(23)   N11)   5' GACGTTCCGGACTACGGTTCTTAATAGAATTCG 3'
(24)   N12)   5' TCGACGAATTCTATTAAGAACCGTAGTC 3'

35

The completed synthetic DNA insert was ligated
directly into the Lambda Zap™ II vector described in
Example 1a1) that had been previously digested with
the restriction enzymes, Not I and Xho I. The

5   ligation mixture was packaged according to the
manufacture's instructions using Gigapack II Gold
packing extract available from Stratagene, La Jolla,
California. The packaged ligation mixture was plated
on XL1-Blue cells (Stratagene). Individual lambda

10   plaques were cored and the inserts excised according
to the in vivo excision protocol for Lambda Zap™ II
provided by the manufacturer (Stratagene). This in
vivo excision protocol moved the cloned insert from
the Lambda Hc2 vector into a phagemid vector to allow

15   easy for manipulation and sequencing. The accuracy of
the above cloning steps was confirmed by sequencing
the insert using the Sanger dideoxy method described
in by Sanger et al., Proc. Natl. Acad. Sci., USA,
74:5463-5467 (1977) and using the manufacture's

20   instructions in the AMV Reverse Transcriptase ^{35}S-ATP
sequencing kit (Stratagene). The sequence of the
resulting double-stranded synthetic DNA insert in the
V_H expression vector (Lambda Hc2) is shown in Figure
1. The sequence of each strand (top and bottom) of

25   Lambda Hc2 is listed in the Sequence Listing as SEQ ID
NO 1 and SEQ ID NO 2, respectively. The resultant
Lambda Hc2 expression vector is shown in Figure 2.

3)   Preparation of Lambda Lc2

30   To express a plurality of V_L-coding DNA
homologs in an E. coli host cell, a vector designated
Lambda Lc2 was constructed having the capacity to
place the V_L-coding DNA homologs in the proper reading
frame, provided a ribosome binding site as described

35   by Shine et al., Nature, 254:34 (1975), provided the

pelB gene leader sequence secretion signal that has
been previously used to successfully secrete Fab
fragments in E. coli by Lei et al., J. Bac., 169:4379
(1987) and Better et al., Science, 240:1041 (1988),

5   and also provided a polynucleotide containing a
restriction endonuclease site for cloning. Lambda Lc2
has been previously described by Huse et al., Science,
246:1275-1281 (1989).

A synthetic DNA sequence containing all of the

10   above features was constructed by designing single
stranded polynucleotide segments of 20-60 bases that
would hybridize to each other and form the double
stranded synthetic DNA sequence shown in Figure 3.
The sequence of each individual single-stranded

15   polynucleotide segment (O1-O8) within the double
stranded synthetic DNA sequence is shown in Table 2.

Polynucleotides 02, 03, 04, 05, 06 and 07 (Table
2) were kinased by adding 1 μl (0.1 μg/μl) of each
polynucleotide and 20 units of T_4 polynucleotide

20   kinase to a solution containing 70 mM Tris-HCl at pH
7.6, 10 mM $MgCl_2$, 5 mM DTT, 10 mM beta-
mercaptoethanol, 500 μg/ml of BSA. The solution was
maintained at 37°C for 30 minutes and the reaction
stopped by maintaining the solution at 65°C for 10

25   minutes. The 20 ng each of the two end polynucleo-.
tides, 01 and 08, were added to the above kinasing
reaction solution together with 1/10 volume of a
solution containing 20.0 mM Tris-HCl at pH 7.4, 2.0 mM
$MgCl_2$ and 15.0 mM sodium chloride (NaCl). This

30   solution was heated to 70°C for 5 minutes and allowed
to cool to room temperature, approximately 25°C, over
1.5 hours in a 500 ml beaker of water. During this
time period all 8 polynucleotides annealed to form the
double stranded synthetic DNA insert shown in Figure

35   3. The individual polynucleotides were covalently

linked to each other to stabilize the synthetic DNA
insert by adding 40 μl of the above reaction to a
solution containing 50 mM Tris-HCl at pH 7.5, 7 mM
MgCl$_2$, 1 mM DTT, 1 mM ATP and 10 units of T4 DNA

5   ligase. This solution was maintained at 37°C for 30
minutes and then the T4 DNA ligase was inactivated by
maintaining the solution at 65°C for 10 minutes. The
end polynucleotides were kinased by mixing 52 μl of
the above reaction, 4 μl of a solution containing 10

10   mM ATP and 5 units of T4 polynucleotide kinase. This
solution was maintained at 37°C for 30 minutes and
then the T4 polynucleotide kinase was inactivated by
maintaining the solution at 65°C for 10 minutes.

15   Table 2

SEQ

ID NO

(25)   01)   5' TGAATTCTAAACTAGTCGCCAAGGAGACAGTCAT 3'

(26)   02)   5' AATGAAATACCTATTGCCTACGGCAGCCGCTGGATT 3'

20   (27)   03)   5' GTTATTACTCGCTGCCCAACCAGCCATGGCC 3'

(28)   04)   5' GAGCTCGTCAGTTCTAGAGTTAAGCGGCCG 3'

(29)   05)   5' GTATTTCATTATGACTGTCTCCTTGGCGACTAGTTTAGAA-
                  TTCAAGCT 3'

(30)   06)   5' CAGCGAGTAATAACAATCCAGCGGCTGCCGTAGGCAATAG 3'

25   (31)   07)   5' TGACGAGCTCGGCCATGGCTGGTTGGG 3'

(32)   08)   5' TCGACGGCCGCTTAACTCTAGAAC 3'

The completed synthetic DNA insert was ligated

30   directly into the Lambda Zap™ II vector described in
Example 1a1) that had been previously digested with
the restriction enzymes Sac I and Xho I. The ligation
mixture was packaged according to the manufacture's
instructions using Gigapack II Gold packing extract

35   (Stratagene). The packaged ligation mixture was

plated on XL1-Blue cells (Stratagene). Individual
lambda plaques were cored and the inserts excised
according to the in vivo excision protocol for Lambda
Zap™ II provided by the manufacturer (Stratagene).

5   This in vivo excision protocol moved the cloned insert
from the Lambda Lc2 vector into a plasmid phagemid
vector allow for easy manipulation and sequencing.
The accuracy of the above cloning steps was confirmed
by sequencing the insert using the manufacture's

10   instructions in the AMV Reverse Transcriptase ^{35}S-dATP
sequencing kit (Stratagene). The sequence of the
resulting Lc2 expression vector (Lambda Lc2) is shown
in Figure 3. Each strand is separately listed in the
Sequence Listing as SEQ ID NO 3 and SEQ ID NO 4. The

15   resultant Lc2 vector is schematically diagrammed in
Figure 4.

       A preferred vector for use in this invention,
designated Lambda Lc3, is a derivative of Lambda Lc2
prepared above. Lambda Lc2 contains a Spe I

20   restriction site located 3' to the EcoR I restriction
site and 5' to the Shine-Dalgarno ribosome binding
site as shown in the sequence in Figure 3 and in SEQ
ID NO 3. A Spe I restriction site is also present in
Lambda Hc2 as shown in Figures 1 and 2 and in SEQ ID

25   NO 1. A combinatorial vector, designated pComb, was
constructed by combining portions of Lambda Hc2 and
Lc2 together as described in Example 1a4) below. The
resultant combinatorial pComb vector contained two Spe
I restriction sites, one provided by Lambda Hc2 and

30   one provided by Lambda Lc2, with an EcoR I site in
between. Despite the presence of two Spe I
restriction sites, DNA homologs having Spe I and EcoR
I cohesive termini were successfully directionally
ligated into a pComb expression vector previously

35   digested with Spe I and EcoR I as described in Example

1b below. The proximity of the EcoR I restriction
site to the 3' Spe I site, provided by the Lc2 vector,
inhibited the complete digestion of the 3' Spe I site.
Thus, digesting pComb with Spe I and EcoR I did not
5   result in removal of the EcoR I site between the two
Spe I sites.

    The presence of a second Spe I restriction site
may be undesirable for ligations into a pComb vector
digested only with Spe I as the region between the two
10   sites would be eliminated. Therefore, a derivative of
Lambda Lc2 lacking the second or 3' Spe I site,
designated Lambda Lc3, was produced by first digesting
Lambda Lc2 with Spe I to form a linearized vector.
The ends were filled in to form blunt ends which are
15   ligated together to result in Lambda Lc3 lacking a Spe
I site. Lambda Lc3 is a preferred vector for use in
constructing a combinatorial vector as described
below.

20   4)   Preparation of pComb
         Phagemids were excised from the
expression vectors Lambda Hc2 or Lambda Lc2 using an
in vivo excision protocol described above. Double
stranded DNA was prepared from the phagemid-containing
25   cells according to the methods described by Holmes et
al., Anal. Biochem., 114:193 (1981). The phagemids
resulting from in vivo excision contained the same
nucleotide sequences for antibody fragment cloning and
expression as did the parent vectors, and are
30   designated phagemid Hc2 and Lc2, corresponding to
Lambda Hc2 and Lc2, respectively.

    For the construction of combinatorial phagemid
vector pComb, produced by combining portions of
phagemid Hc2 and phagemid Lc2, phagemid Hc2 was first
35   digested with Sac I to remove the restriction site

located 5' to the LacZ promoter. The linearized
phagemid was then blunt ended with T4 polymerase and
ligated to result in a Hc2 phagemid lacking a Sac I
site. The modified Hc2 phagemid and the Lc2 phagemid

5   were then separately restriction digested with Sca I
and EcoR I to result in a Hc2 fragment having from 5'
to 3' Sca I, Not I, Xho I, Spe I and EcoR I
restriction sites and a Lc2 fragment having from 5' to
3' EcoR I, Sac I, Xba I and Sac I restriction sites.

10   The linearized phagemids were then ligated together at
their respective cohesive ends to form pComb, a
circularized phagemid having a linear arrangement of
restriction sites of Not I, Xho I, Spe I, EcoR I, Sac
I, Xba I, Not I, Apa I and Sca I. The ligated

15   phagemid vector was then inserted into an appropriate
bacterial host and transformants were selected on the
antibiotic ampicillin.

        Selected ampicillin resistant transformants were
screened for the presence of two Not I sites. The

20   resulting ampicillin resistant combinatorial phagemid
vector was designated pComb, the schematic
organization of which is shown in Figure 5. The
resultant combinatorial vector, pComb, consisted of a
DNA molecule having two cassettes to express two

25   fusion proteins and having nucleotide residue
sequences for the following operatively linked
elements listed in a 5' to 3' direction: a first
cassette consisting of an inducible LacZ promoter
upstream from the LacZ gene; a Not I restriction site;

30   a ribosome binding site; a pelB leader; a spacer; a
cloning region bordered by a 5' Xho and 3' Spe I
restriction site; a decapeptide tag followed by
expression control stop sequences; an EcoR I
restriction site located 5' to a second cassette

35   consisting of an expression control ribosome binding

site; a pelB leader; a spacer region; a cloning region
bordered by a 5' Sac I and a 3' Xba I restriction site
followed by expression control stop sequences and a
second Not I restriction site.

5   A preferred combinatorial vector for use in this
invention, designated pComb2, is constructed by
combining portions of phagemid Hc2 and phagemid Lc3 as
described above for preparing pComb. The resultant
combinatorial vector, pComb2, consists of a DNA

10   molecule having two cassettes identical to pComb to
express two fusion proteins identically to pComb
except that a second Spe I restriction site in the
second cassette is eliminated.

15   b.   Construction of the pCombIII Vector for
                Expressing Fusion Proteins Having a
                Bacteriophage Coat Protein Membrane Anchor
                Because of the multiple endonuclease
restriction cloning sites, the pComb phagemid
expression vector prepared above is a useful cloning

20   vehicle for modification for the preparation of an
expression vector for use in this invention. To that
end, pComb was digested with EcoR I and Spe I followed
by phosphatase treatment to produce linearized pComb.

25

                1)   Preparation of pCombIII
                A separate phagemid expression vector
was constructed using sequences encoding bacteriophage
cpIII membrane anchor domain. A PCR product defining

30   the DNA sequence encoding the filamentous phage coat
protein,cpIII, membrane anchor containing a LacZ
promotor region sequence 3' to the membrane anchor for
expression of the light chain and Spe I and EcoR I
cohesive termini was prepared from M13mp18, a

35   commercially available bacteriophage vector

(Pharmacia, Piscataway, New Jersey).

   To prepare a modified cpIII, replicative form DNA
from M13mp18 was first isolated. Briefly, into 2 ml
of LB (Luria-Bertani medium), 50 μl of a culture of a
bacterial strain carrying an F' episome (JM107, JM109
or TG1) was admixed with a one tenth suspension of
bacteriophage particles derived from a single plaque.
The admixture was incubated for 4 to 5 hours at 37°C
with constant agitation. The admixture was then
centrifuged at 12,000 x g for 5 minutes to pellet the
infected bacteria. After the supernatant was removed,
the pellet was resuspended by vigorous vortexing in
100 μl of ice-cold solution I. Solution I was
prepared by admixing 50 mM glucose, 10 mM EDTA
(disodium ethylenediaminetetraacetic acid) and 25 mM
Tris-HCl at pH 8.0, and autoclaving for 15 minutes.

   To the bacterial suspension, 200 μl of freshly
prepared Solution II was admixed and the tube was
rapidly inverted five times. Solution II was prepared
by admixing 0.2 N NaOH and 1% SDS. To the bacterial
suspension, 150 μl of ice-cold Solution III was
admixed and the tube was vortexed gently in an
inverted position for 10 seconds to disperse Solution
III through the viscous bacterial lysate. Solution
III was prepared by admixing 60 ml of 5 M potassium
acetate, 11.5 ml of glacial acetic acid and 28.5 ml of
water. The resultant bacterial lysate was then stored
on ice for 5 minutes followed by centrifugation at
12,000 x g for 5 minutes at 4°C in a microfuge. The
resultant supernatant was recovered and transferred to
a new tube. To the supernatant was added an equal
volume of phenol/chloroform and the admixture was
vortexed. The admixture was then centrifuged at
12,000 x g for 2 minutes in a microfuge. The
resultant supernatant was transferred to a new tube

and the double-stranded bacteriophage DNA was
precipitated with 2 volumes of ethanol at room
temperature. After allowing the admixture to stand at
room temperature for 2 minutes, the admixture was
centrifuged to pellet the DNA. The supernatant was
removed and the pelleted replicative form DNA was
resuspended in 25 μl of Tris-HCl at pH 7.6, and 10 mM
EDTA (TE).

An alternative Lac-B primer for use in
constructing the cpIII membrane anchor and LacZ
promotor region was Lac-B' as shown in Table 3. The
amplification reactions were performed as described
above with the exception that in the second PCR
amplification, Lac-B' was used with Lac-F instead of
Lac-B. The product from the amplification reaction is
listed in the sequence listing as SEQ ID NO 41 from
nucleotide position 1 to nucleotide position 172. The
use of Lac-B' resulted in a LacZ region lacking 29
nucleotides on the 3' end but was functionally
equivalent to the longer fragment produced with the
Lac-F and Lac-B primers.

The products of the first and second PCR
amplifications using the primer pairs G-3(F) and
G-3(B) and Lac-F and Lac-B were then recombined at the
nucleotides corresponding to cpIII membrane anchor
overlap and Nhe I restriction site and subjected to a
second round of PCR using the G-3(F) (SEQ ID NO 35)
and Lac-B (SEQ ID NO 38) primer pair to form a
recombined PCR DNA fragment product consisting of the
following: a 5' Spe I restriction site; a cpIII DNA
membrane anchor domain beginning at the nucleotide
residue sequence which corresponds to the amino acid
residue 198 of the entire mature cpIII protein; an
endogenous stop site provided by the membrane anchor

at amino acid residue number 112; a Nhe I restriction
site, a LacZ promoter, operator and Cap-binding site
sequence; and a 3' EcoR I restriction site.

To construct a phagemid vector for the coordinate
expression of a heavy chain-cpIII fusion protein as
prepared in Example 2 with kappa light chain, the
recombined PCR modified cpIII membrane anchor domain
DNA fragment was then restriction digested with Spe I
and EcoR I to produce a DNA fragment for directional
ligation into a similarly digested pComb2 phagemid
expression vector having only one Spe I site prepared
in Example 1a4) to form a pComb2-III (also referred to
as pComb2-III) phagemid expression vector. Thus, the
resultant ampicillin resistance conferring pComb2-3
vector, having only one Spe I restriction site,
contained separate LacZ promoter/operator sequences
for directing the separate expression of the heavy
chain (Fd)-cpIII fusion product and the light chain
protein. The expressed proteins were directed to the
periplasmic space by pelB leader sequences for
functional assembly on the membrane. Inclusion of the
phage F1 intergenic region in the vector allowed for
packaging of single stranded phagemid with the aid of
helper phage. The use of helper phage superinfection
lead to expression of two forms of cpIII. Thus,
normal phage morphogenesis was perturbed by
competition between the Fab-cpIII fusion and the
native cpIII of the helper phage for incorporation
into the virion for Fab-cpVIII fusions. In addition,
also contemplated for use in this invention are
vectors conferring chloramphenicol resistance and the
like.

A more preferred phagemid expression vector for
use in this invention having additional restriction
enzyme cloning sites, designated pComb-III' or

pComb2-3', was prepared as described above for
pComb2-3 with the addition of a 51 base pair fragment
from pBluescript as described by Short et al., <u>Nuc.</u>
<u>Acids Res</u>., 16:7583-7600 (1988) and commercially

5   available from Stratagene. To prepare pComb2-3',
pComb2-3 was first digested with Xho I and Spe I
restriction enzymes to form a linearized pComb2-3.
The vector pBluescript was digested with the same
enzymes releasing a 51 base pair fragment containing

10   the restriction enzyme sites Sal I, Acc I, Hinc II,
Cla I, Hind III, EcoR V, Pst I, Sma I and BamH I. The
51 base pair fragment was ligated into the linearized
pComb2-3 vector via the cohesive Xho I and Spe I
termini to form pComb2-3'.

15
                        Table 3

SEQ

ID NO	Primer		
(35)[1]	G-3	(F)	5' GAGACG<u>ACTAGT</u><u><u>GGTGGCGGTGGCTCTCCATTC</u></u> <u><u>GTTTGTGAATATCAA</u></u> 3'
(36)[2]	G-3	(B)	5' TTACTA<u>GCTAGC</u><u><u>ATAATAACGGAATACCCAAAA</u></u> <u><u>GAACTGG</u></u> 3'
(37)[3]	LAC-F		5' <u>TATGCTAGCTAGTAA</u>CACGACAGGTTTCCCGAC TGG 3'
(38)[4]	LAC-B		5' ACCGAGCTC<u>GAATTC</u>GTAATCATGGTC 3'
(39)5	LAC-B'		5' AGCTGTT<u>GAATTC</u>GTGAAATTGTTATCCGCT 3'

F   Forward Primer

30 B   Backward Primer
1 From 5' to 3': Spe I restriction site sequence is
single underlined; the overlapping sequence with the
5' end of cpIII is double underlined
2 From 5' to 3': Nhe I restriction site sequence is

35 single underlined; the overlapping sequence with 3'

end of cpIII is double underlined.

3 From 5' to 3': overlapping sequence with the 3' end of cpIII is double underlined; Nhe I restriction sequence begins with the nucleotide residue "G" at position 4 and extends 5 more residues = GCTAGC.

4 EcoR I restriction site sequence is single underlined.

5 Alternative backwards primer for amplifying LacZ; EcoR I restriction site sequence is single underlined.

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2.   Isolation of HIV-1-Specific Monoclonal Antibodies Produced from the Dicistronic Expression Vector, pComb2-3

In practicing this invention, the heavy (Fd consisting of V_H and C_H1) and light (kappa) chains (V_L, C_L) of antibodies are first targeted to the periplasm of E. coli for the assembly of heterodimeric Fab molecules. In order to obtain expression of antibody Fab libraries on a phage surface, the nucleotide residue sequences encoding either the Fd or light chains must be operatively linked to the nucleotide residue sequence encoding a filamentous bacteriophage coat protein membrane anchor. A coat protein for use in this invention in providing a membrane anchor is III (cpIII or cp3). In the Examples described herein, methods for operatively linking a nucleotide residue sequence encoding a Fd chain to a cpIII membrane anchor in a fusion protein of this invention are described.

In a phagemid vector, a first and second cistron consisting of translatable DNA sequences are operatively linked to form a dicistronic DNA molecule. Each cistron in the dicistronic DNA molecule is linked to DNA expression control sequences for the coordinate

expression of a fusion protein, Fd-cpIII, and a kappa light chain.

The first cistron encodes a periplasmic secretion signal (pelB leader) operatively linked to the fusion protein, Fd-cpIII. The second cistron encodes a second pelB leader operatively linked to a kappa light chain. The presence of the pelB leader facilitates the coordinated but separate secretion of both the fusion protein and light chain from the bacterial cytoplasm into the periplasmic space.

In this process, the phagemid expression vector carries an ampicillin selectable resistance marker gene (beta lactamase or bla) in addition to the Fd-cpIII fusion and the kappa chain. The f1 phage origin of replication facilitates the generation of single stranded phagemid. The isopropyl thiogalactopyranoside (IPTG) induced expression of a dicistronic message encoding the Fd-cpIII fusion (V_H, C_{H1}, cpIII) and the light chain (V_L, C_L) leads to the formation of heavy and light chains. Each chain is delivered to the periplasmic space by the pelB leader sequence, which is subsequently cleaved. The heavy chain is anchored in the membrane by the cpIII membrane anchor domain while the light chain is secreted into the periplasm. The heavy chain in the presence of light chain assembles to form Fab molecules. This same result can be achieved if, in the alternative, the light chain is anchored in the membrane via a light chain fusion protein having a membrane anchor and heavy chain is secreted via a pelB leader into the periplasm.

With subsequent infection of E. coli with a helper phage, as the assembly of the filamentous bacteriophage progresses, the coat protein III is incorporated on the tail of the bacteriophage.

a.   Preparation of Lymphocyte RNA

Five milliliters of bone marrow was removed
by aspiration from HIV-1 asymptomatic seropositive
individuals. Total cellular RNA was prepared from the
5   bone marrow lymphocytes as described above using the
RNA preparation methods described by Chomczynski et
al., Anal Biochem., 162:156-159 (1987) and using the
RNA isolation kit (Stratagene) according to the
manufacturer's instructions. Briefly, for immediate
10   homogenization of the cells in the isolated bone
marrow, 10 ml of a denaturing solution containing 3.0
M guanidinium isothiocyanate containing 71 μl of
beta-mercaptoethanol was admixed to the isolated bone
marrow. One ml of sodium acetate at a concentration
15   of 2 M at pH 4.0 was then admixed with the homogenized
cells. One ml of phenol that had been previously
saturated with H_2O was also admixed to the denaturing
solution containing the homogenized spleen. Two ml of
a chloroform:isoamyl alcohol (24:1 v/v) mixture was
20   added to this homogenate. The homogenate was mixed
vigorously for ten seconds and maintained on ice for
15 minutes. The homogenate was then transferred to a
thick-walled 50 ml polypropylene centrifuged tube
(Fisher Scientific Company, Pittsburgh, PA). The
25   solution was centrifuged at 10,000 x g for 20 minutes
at 4°C. The upper RNA-containing aqueous layer was
transferred to a fresh 50 ml polypropylene centrifuge
tube and mixed with an equal volume of isopropyl
alcohol. This solution was maintained at -20°C for at
30   least one hour to precipitate the RNA. The solution
containing the precipitated RNA was centrifuged at
10,000 x g for twenty minutes at 4°C. The pelleted
total cellular RNA was collected and dissolved in 3 ml
of the denaturing solution described above. Three ml
35   of isopropyl alcohol was added to the re-suspended

total cellular RNA and vigorously mixed. This
solution was maintained at -20°C for at least 1 hour
to precipitate the RNA. The solution containing the
precipitated RNA was centrifuged at 10,000 x g for ten

5   minutes at 4°C. The pelleted RNA was washed once with
a solution containing 75% ethanol. The pelleted RNA
was dried under vacuum for 15 minutes and then
re-suspended in dimethyl pyrocarbonate-treated
(DEPC-H$_2$O) H$_2$O.

10   Messenger RNA (mRNA) enriched for sequences
containing long poly A tracts was prepared from the
total cellular RNA using methods described in
Molecular Cloning: A Laboratory Manual, Maniatis et
al., eds., Cold Spring Harbor, NY, (1982). Briefly,

15   one half of the total RNA isolated from a single donor
prepared as described above was resuspended in one ml
of DEPC-H$_2$O and maintained at 65°C for five minutes.
One ml of 2X high salt loading buffer consisting of
100 mM Tris-HCl, 1 M NaCl, 2.0 mM EDTA at pH 7.5, and

20   0.2% SDS was admixed to the resuspended RNA and the
mixture allowed to cool to room temperature.
     The total purified mRNA was then used in PCR
amplification reactions as described in Example 2c.
Alternatively, the mRNA was further purified to poly

25   A+ RNA by the following procedure. The total MRNA was
applied to an oligo-dT (Collaborative Research Type 2
or Type 3) column that was previously prepared by
washing the oligo-dT with a solution containing 0.1 M
sodium hydroxide and 5 mM EDTA and then equilibrating

30   the column with DEPC-H$_2$O. The eluate was collected in
a sterile polypropylene tube and reapplied to the same
column after heating the eluate for 5 minutes at 65°C.
The oligo-dT column was then washed with 2 ml of high
salt loading buffer consisting of 50 mM Tris-HCl at pH

35   7.5, 500 mM sodium chloride, 1 mM EDTA at pH 7.5 and

0.1% SDS. The oligo dT column was then washed with 2
ml of 1X medium salt buffer consisting of 50 mM
Tris-HCl, pH 7.5, 100 mM, 1 mM EDTA and 0.1% SDS. The
messenger RNA was eluted from the oligo-dT column with

5   1 ml of buffer consisting of 10 mM Tris-HCl at pH 7.5,
1 mM EDTA at pH 7.5, and 0.05% SDS. The messenger RNA
was purified by extracting this solution with
phenol/chloroform followed by a single extraction with
100% chloroform. The messenger RNA was concentrated

10   by ethanol precipitation and resuspended in DEPC H_2O.

The resultant purified mRNA contained a plurality
of anti-HIV encoding V_H and V_L sequences for
preparation of an anti-HIV-1 Fab DNA library.

15   b.   Construction of a Combinatorial HIV-1
Antibody Library
1)   Selection of Oligonucleotide Primers
The nucleotide sequences encoding the
immunoglobulin protein CDR's are highly variable.
20   However, there are several regions of conserved
sequences that flank the V region domains of either
the light or heavy chain, for instance, and that
contain substantially conserved nucleotide sequences,
i.e., sequences that will hybridize to the same primer

25   sequence. Therefore, polynucleotide synthesis
(amplification) primers that hybridize to the
conserved sequences and incorporate restriction sites
into the DNA homolog produced that are suitable for
operatively linking the synthesized DNA fragments to a

30   vector were constructed. More specifically, the
primers were designed so that the resulting DNA
homologs produced can be inserted into an expression
vector of this invention in reading frame with the
upstream translatable DNA sequence at the region of

35   the vector containing the directional ligation means.

For amplification of the V_H domains, primers were
designed to introduce cohesive termini compatible with
directional ligation into the unique Xho I and Spe I
sites of the pComb2-3 expression vector. In all

5   cases, the 5′ primers VH1a (5′
CAGGTGCAG<u>CTCGAG</u>CAGTCTGGG 3′ SEQ ID NO 42) and VH3a (5′
GAGGTGCAG<u>CTCGAG</u>GAGTCTGGG 3′ SEQ ID NO 43) were
designed to maximize homology with the V_H1 and V_H3
subgroup families, respectively, although considerable

10   cross-priming of other subgroups was expected. The
Xho I restriction site for cloning into the pComb2-3
vector is underlined. The 3′ primer CG1z having the
nucleotide sequence 5′ GCATGT<u>ACTAGT</u>TTTGTCACAAGATTTGGG
3′ (SEQ ID NO 44) used in conjunction with the 5′

15   primers is the primer for the heavy chain
corresponding to part of the hinge region. The Spe I
site for cloning into the pComb2-3 vector is
underlined.

The nucleotide sequences encoding the V_L domain

20   are highly variable. However, there are several
regions of conserved sequences that flank the V_L
domains including the J_L, V_L framework regions and V_L
leader/promotor. Therefore, amplification primers
were constructed that hybridized to the conserved

25   sequences and incorporate restriction sites that allow
cloning the amplified fragments into the pComb2-3
expression vector cut with Sac I and Xba I.

For amplification of the kappa V_L domains
analogous to the heavy chain primers listed above, the

30   5′ primers, VK1a (5′ GACATC<u>GAGCTC</u>ACCCAGTCTCCA 3′ SEQ
ID NO 45) and VK3a (5′ GAAATT<u>GAGCTC</u>ACGCAGTCTCCA 3′ SEQ
ID NO 46), were used. These primers also introduced a
Sac I restriction endonuclease site indicated by the
underlined nucleotides to allow the V_L DNA homolog to

35   be cloned into the pComb2-3 expression vector. The 3′

V_L amplification primer, CK1a having a nucleotide sequence 5' GCGCCG<u>TCTAGA</u>ACTAACACTCTCCCCTGTTGAAGCTCTTTGTGACGGGCAAG 3' (SEQ ID NO 47) corresponding to the 3' end of the light chain was used to amplify the light chain while incorporating the underlined Xba I restriction endonuclease site required to insert the V_L DNA homolog into the pComb2-3 expression vector.

All primers and synthetic polynucleotides described herein, were either purchased from Research Genetics in Huntsville, Alabama or synthesized on an Applied Biosystems DNA synthesizer, model 381A, using the manufacturer's instruction.

2)   PCR Amplification of V_H and V_L DNA Homologs

In preparation for PCR amplification, mRNA prepared above was used as a template for cDNA synthesis by a primer extension reaction. First, 20-50 μg of total mRNA in water was first hybridized (annealed) at 70°C for 10 minutes with 600 ng (60.0 pmol) of either the heavy or light chain 3' primers listed above. Subsequently, the hybridized admixture was used in a typical 50 μl reverse transcription reaction containing 200 μM each of dATP, dCTP, dGTP and dTTP, 40 mM Tris-HCl at pH 8.0, 8 mM $MgCl_2$, 50 mM NaCl, 2 mM spermidine and 600 units of reverse transcriptase (SuperScript, BRL). The reaction admixture was then maintained for one hour at 37°C to form an RNA-cDNA admixture.

Three μl of the resultant RNA-cDNA admixture was then used in PCR amplification in a reaction volume of 100 μl containing a mixture of all four dNTPs at a concentration of 60 μM, 50 mM KCl, 10 mM Tris-HCl at pH 8.3, 15 mM $MgCl_2$, 0.1% gelatin and 5 units of

Thermus aquaticus (Taq) DNA polymerase (Perkin-Elmer-Cetus, Emeryville, California), and 60 pmol of the appropriate 5' and 3' primers listed above. The separate reaction admixtures were overlaid with

5   mineral oil and subjected to 35 cycles of amplification. Each amplification cycle included denaturation at 91°C for 1 minute, annealing at 52°C for 2 minutes and polynucleotide synthesis by primer extension (elongation) at 72°C for 1.5 minutes,

10 followed by a final maintenance period of 10 minutes at 72°C. An aliquot of the reaction admixtures were then separately electrophoresed on a 2% agarose gel. After successful amplification as determined by gel electrophoretic migration, the remainder of the

15 RNA-cDNA was amplified after which the PCR products of a common 3' primer were pooled into separate V_H-and V_L-coding DNA homolog-containing samples and were then extracted twice with phenol/chloroform, once with chloroform, ethanol precipitated and were stored at

20 -70°C in 10 mM Tris-HCl at pH 7.5, and 1 mM EDTA.

3)   Insertion of V_H and V_L-Coding DNA
     Homologs into pComb2-3 Expression
     Vector

25   The V_H-coding DNA homologs (heavy chain) prepared above were then digested with an excess of Xho I and Spe I for subsequent ligation into a similarly digested and linearized pComb2-3 in a total volume of 150 μl with 10 units of ligase at 16°C

30 overnight. The construction of the library was performed as described by Burton et al., Proc. Natl. Acad. Sci., USA, 88:10134-10137 (1991). Briefly, following ligation, the pComb2-3 vector containing heavy chain DNA was then transformed by

35 electroporation into 300 μl of XL1-Blue cells. After

transformation and culturing, library size was
determined by plating aliquots of the culture.
Typically the library had about 10^7 members. An
overnight culture was then prepared from which

5   phagemid DNA containing the heavy chain library was
prepared.

For the cloning of the V_L-coding DNA homologs
(light chain), 10 μg of phagemid DNA containing the
heavy chain library was then digested with Sac I and

10   SbaI. The resulting linearized vector was treated
with phosphatase and purified by agarose gel
electrophoresis. The desired fragment, 4.7 kb in
length, was excised from the gel. Ligation of this
vector with prepared light chain PCR DNA proceeded as

15   described above for heavy chain. A library of
approximately 10^7 members having heavy chain fragments
operatively linked to the cpIII anchor sequence
(Fd-cpIII) and light chain fragments was thus
produced.

20

4)   Preparation of Phage Expressing Fab
Heterodimers
Following transformation of the
resultant library produced above into XL1-Blue cells,

25   phage were prepared to allow for isolation of HIV-1
specific Fabs by panning on target antigens. To
isolate phage on which heterodimer expression has been
induced, 3 ml of SOC medium (SOC was prepared by
admixture of 20 g bacto-tryptone, 5 g yeast extract

30   and 0.5 g NaCl in one liter of water, adjusting the pH
to 7.5 and admixing 20 ml of glucose just before use
to induce the expression of the Fd-cpIII and light
chain heterodimer) was admixed and the culture was
shaken at 220 rpm for one hour at 37°C, after which

35   time 10 ml of SB (SB was prepared by admixing 30 g

tryptone, 20 g yeast extract, and 10 g Mops buffer per
liter with pH adjusted to 7) containing 20 μg/ml
carbenicillin and 10 μg/ml tetracycline and the
admixture was shaken at 300 rpm for an additional

5   hour. This resultant admixture was admixed to 100 ml
SB containing 50 μg/ml carbenicillin and 10 μg/ml
tetracycline and shaken for one hour, after which time
helper phage VCSM13 (10^{12} pfu) were admixed and the
admixture was shaken for an additional two hours.

10   After this time, 70 μg/ml kanamycin was admixed and
maintained at 30°C overnight. The lower temperature
resulted in better heterodimer incorporation on the
surface of the phage. The supernatant was cleared by
centrifugation (4000 rpm for 15 minutes in a JA10

15   rotor at 4°C). Phage were precipitated by admixture
of 4% (w/v) polyethylene glycol 8000 and 3% (w/v) NaCl
and maintained on ice for 30 minutes, followed by
centrifugation (9000 rpm for 20 minutes in a JA10
rotor at 4°C). Phage pellets were resuspended in 2 ml

20   of PBS and microcentrifuged for three minutes to
pellet debris, transferred to fresh tubes and stored
at -20°C for subsequent screening as described below.

          For determining the titering colony forming units
(cfu), phage (packaged phagemid) were diluted in SB

25   and 1 μl was used to infect 50 μl of fresh (OD600 = 1)
XL1-Blue cells grown in SB containing 10 μg/ml
tetracycline. Phage and cells were maintained at room
temperature for 15 minutes and then directly plated on
LB/carbenicillin plates.

30

                5)   Selection of Anti-HIV-1 Heterodimers on
                      Phage Surfaces
                (a)   Multiple Pannings of the Phage
                       Library

35   The phage library produced in

Example 2b4) was panned against recombinant gp120 of
HIV-1 strain IIIb as described herein on coated
microtiter plate to select for anti-gp120
heterodimers. A second phage library was panned

5   against recombinant gp41 (American Biotechnologies,
Boston, MA) as described below to select for anti-gp41
heterodimers.

The panning procedure used was a modification of
that originally described by Parmley and Smith

10   (Parmley et al., Gene, 73:305-318 (1988). Four rounds
of panning were performed to enrich for specific
antigen-binding clones. For this procedure, four
wells of a microtiter plate (Costar 3690) were coated
overnight at 4°C with 25 μl of 40 μg/ml gp120 or gp41

15   (American Biotechnologies) prepared above in 0.1 M
bicarbonate, pH 8.6. The wells were washed twice with
water and blocked by completely filling the well with
3% (w/v) BSA in PBS and maintaining the plate at 37°C
for one hour. After the blocking solution was shaken

20   out, 50 μl of the phage library prepared above
(typically 10^{11} cfu) were admixed to each well, and
the plate was maintained for two hours at 37°C.

Phage were removed and the plate was washed once
with water. Each well was then washed ten times with

25   TBS/Tween (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.5%
Tween 20) over a period of one hour at room
temperature where the washing consisted of pipetting
up and down to wash the well, each time allowing the
well to remain completely filled with TBS/Tween

30   between washings. The plate was washed once more with
distilled water and adherent phage were eluted by the
addition of 50 μl of elution buffer (0.1 M HCl,
adjusted to pH 2.2 with solid glycine, containing 1
mg/ml BSA) to each well followed by maintenance at

35   room temperature for 10 minutes. The elution buffer

was pipetted up and down several times, removed, and neutralized with 3 μl of 2 M Tris base per 50 μl of elution buffer used.

Eluted phage were used to infect 2 ml of fresh (OD$_{600}$ = 1) E. coli XL1-Blue cells for 15 minutes at room temperature, after which time 10 ml of SB containing 20 μg/ml carbenicillin and 10 μg/ml tetracycline was admixed. Aliquots of 20, 10, and 1/10 μl were removed from the culture for plating to determine the number of phage (packaged phagemids) that were eluted from the plate. The culture was shaken for one hour at 37°C, after which it was added to 100 ml of SB containing 50 μg/ml carbenicillin and 10 μg/ml tetracycline and shaken for one hour. Helper phage VCSM13 (10^{12} pfu) were then added and the culture was shaken for an additional two hours. After this time, 70 μg/ml kanamycin was added and the culture was incubated at 37°C overnight. Phage preparation and further panning were repeated as described above.

Following each round of panning, the percentage yield of phage were determined, where % yield - (number of phage eluted/number of phage applied) X 100. The initial phage input ratio was determined by titering on selective plates to be approximately 10^{11} cfu for each round of panning. The final phage output ratio was determined by infecting two ml of logarithmic phase XL1-Blue cells as described above and plating aliquots on selective plates. In the first panning for gp120-reactive phage, 4.6 X 10^{11} phage were applied to four wells and 7.7 X 10^5 phage were eluted. After the fourth panning 1.0 X 10^8 phage were eluted. From this procedure, 20 clones were selected from the Fab library for their ability to bind to glycosylated recombinant gp120 from the IIIB

strain of HIV-1. Five clones were selected from the
Fab library specific for binding to gp41. The panned
phage surface libraries were then converted into ones
expressing soluble Fab fragments for further screening
5   by ELISA as described below.

In addition to panning on gp120 of strain IIIB
and gp41, also contemplated as antigens for panning of
combinatorial libraries is recombinant gp120 (IIIB
strain) produced in baculovirus and recombinant gp120
10   (SF2 strain) produced in Chinese Hamster Ovary cells
obtained as described by Steimer et al., Science,
254:105-108 (1991). Another antigen, a synthetic
cyclic peptide, $N=CH-(CH_2)_3CO[SISGPGRAFYTG]NCH_2CO-Cys-NH_2$ (SEQ ID NO 48) prepared as described by
15   Satterthwait et al., Bulletin of the World Health
Organization, 68: Suppl., 17-25 (1990) corresponding
to the central most conserved part of the V3 loop of
gp120 was coupled to maleimide-activated BSA. The
library was panned using 1, 2 or 4 ELISA wells coated
20   with 1 μg of protein antigen or 10 μg BSA-peptide per
well. Four rounds of panning were carried out for
each antigen as described above. Eluted phage from
the final round were used to infect XL1-Blue cells.
Four rounds of panning against the four antigens
25   produced an amplification in eluted phage of between
100 and 1000 fold. The panned phage surface libraries
were then converted into ones expressing soluble Fab
fragments for further screening by ELISA as described
below.

30

6)   Preparation of Soluble Heterodimers and
Characterization of Binding Specificity
to HIV-1 Antigens

In order to further characterize the
35   specificity of the mutagenized heterodimers expressed

on the surface of phage as described above, soluble
Fab heterodimers from acid eluted phage were prepared
and analyzed in ELISA assays on HIV-1 derived
antigen-coated plates and by competitive ELISA.

5   To prepare soluble heterodimers, phagemid DNA
from the 20 gp120 positive clones and the 5 gp41
positive clones prepared above was isolated and
digested with Spe I and Nhe I. Digestion with these
enzymes produced compatible cohesive ends. The 4.7 kb

10   DNA fragment lacking the gene III portion was
gel-purified (0.6% agarose) and self-ligated.
Transformation of E. coli XL1-Blue afforded the
isolation of recombinants lacking the cpIII fragment.
Clones were examined for removal of the cpIII fragment

15   by Xho I - Xba I digestion, which should yield an
1.6-kb fragment. Clones were grown in 100 ml SB
containing 50 μg/ml carbenicillin and 20 mM MgCl$_2$ at
37°C until an OD$_{600}$ of 0.2 was achieved. IPTG (1 mM)
was added and the culture grown overnight at 30°C

20   (growth at 37°C provides only a light reduction in
heterodimer yield). Cells were pelleted by
centrifugation at 4000 rpm for 15 minutes in a JA10
rotor at 4°C. Cells were resuspended in 4 ml PBS
containing 34 μg/ml phenylmethylsulfonyl fluoride

25   (PMSF) and lysed by sonication on ice (2-4 minutes at
50% duty). Debris was pelleted by centrifugation at
14,000 rpm in a JA20 rotor at 4°C for 15 minutes. The
supernatant was used directly for ELISA analysis as
described below and was stored at -20°C. For the

30   study of a large number of clones, 10 ml cultures
provided sufficient heterodimer for analysis. In this
case, sonications were performed in 2 ml of buffer.

        Assays as described above were also performed for
the gp41-specific clones.

35

a)   Screening by ELISA

The soluble heterodimers prepared
above were assayed by ELISA. For this assay, gp120
and gp41 were separately admixed to individual wells
of a microtiter plate as described above for the
panning procedure and maintained at 4°C overnight to
allow the protein solution to adhere to the walls of
the well. After the maintenance period, the wells
were washed five times with water and thereafter
maintained for one hour at 37°C with 100 μl solution
of 1% BSA diluted in PBS to block nonspecific sites on
the wells. Afterwards, the plates were inverted and
shaken to remove the BSA solution. Twenty-five μl of
soluble heterodimers prepared above reactive with the
specific glycoprotein substrate were then admixed to
each well and maintained at 37°C for one hour to form
immunoreaction products. Following the maintenance
period, the wells were washed ten times with water to
remove unbound soluble antibody and then maintained
with a 25 μl of a 1:1000 dilution of secondary goat
anti-human IgG F(ab')$_2$ conjugated to alkaline
phosphatase diluted in PBS containing 1% BSA. The
wells were maintained at 37°C for one hour after which
the wells were washed ten times with water followed by
development with 50 μl of p-nitrophenyl phosphate
(PNPP). Color development was monitored at 405 nm.
Positive clones gave A405 values of >1 (mostly >1.5)
after 10 minutes, whereas negative clones gave values
of 0.1 to 0.2.

Approximate concentrations of gp120-reactive Fab
were determined by ELISA using a sandwich ELISA as
described by Zebedee et al., Proc. Natl. Acad. Sci.,
USA, 89:3175-3179 (1992) and are presented in the
first column of Figure 6. In addition, since Fabs are
expressed in E. coli and the fraction of correctly

assemble protein can vary, the amount of Fab reacting with gp120 was also assessed by ELISA titration. That data is also presented in Figure 6 in the second column.

5   For the clones panned against the HIV-1 derived antigens, after conversion of the panned phage surface libraries to ones expressing soluble Fab fragments, 30-40 colonies were used to transform XL1-Blue cells and the supernates screened in ELISA assays against

10   the antigen used in panning. Generally greater than 80% of the supernates tested positive. A representative number of positives were then selected from each antigen panning for further analysis.

15   (b)   Competitive ELISA with Soluble
                        gp120 and CD4

Immunoreactive heterodimers as determined in the above ELISA were then analyzed by competition ELISA to determine the affinity of the

20   selected heterodimers. The ELISA was performed as described above on microtiter wells separately coated with 5 μg/ml of gp120 or soluble CD4 (American Biotechnologies) in 0.1 M bicarbonate buffer at pH 8.6. Increasing concentrations of soluble or free

25   gp120 ranging in concentration from 10^{-11} M up to 10^{-7} M diluted in 0.5% BSA/0.025% Tween 20/PBS were admixed with soluble heterodimers, the dilutions of which were determined in titration experiments that resulted in substantial reduction of OD values after a 2-fold

30   dilution. For the CD4 competition assays, increasing concentrations of soluble or free CD4 ranging in concentration from 10^{-11} M up to 10^{-6} M diluted in 0.5% BSA/0.025% Tween 20/PBS were admixed with soluble heterodimers. The plates were maintained for 90-120

35   minutes at 37°C and carefully washed ten times with

0.05% Tween 20/PBS before admixture of alkaline
phosphatase-labelled goat anti-human IgG F(ab')2 at a
dilution of 1:500 followed by maintenance for 1 hour
at 37°C.   Development was performed as described for
5   ELISA.

     To establish the relationship between
neutralizing ability as described in Example 3 below
could be related to antigen binding affinity of
HIV-1-specific Fabs, competition ELISAs were carried
10   out where soluble gp120 was competed with gp120 coated
on ELISA plates for Fab binding.   Figure 7 shows that
all Fabs were competed from binding to gp120 with a
IC_{50} of approximately 10^{-9} M free gp120.   In addition
as shown in Example 3, there is no correlation between
15   antigen affinity and neutralization.   The Fabs tested
included Fabs 4, 12, 21 and 7 that are members of the
same groups as determined by sequence analysis and
comparison as described in Example 9.   Fabs 13, 27, 6,
29, 2 and 3 are all members of the different groups as
20   determined by sequence analysis and comparison as
described in Example 9.   Loop 2 is an Fab fragment
selected from the same library as the other Fabs but
which recognizes the V3 loop.   Only with the V3 loop
peptide was competition carried out with gp120 from
25   the SF2 strain.

     To investigate whether neutralization could be
associated with blocking of the gp120-CD4 interaction,
competition ELISAs were carried out with soluble CD4
competing with Fabs for binding to gp120-coated ELISA
30   wells.   The results are shown in Figure 8.   P4D10 and
loop 2 are controls not expected to be competed by
CD4.   P4D10 is a mouse monoclonal antibody reacting
with the V3 loop of gp120 (IIIB).   Loop 2 Fab
competition was carried out using gp120 (SF2).   As
35   shown in Figure 8 the binding of all Fabs with the

exception of the controls was inhibited with an IC_{50} of approximately 10^{-8} M of soluble CD4. In addition, no difference was detected between the neutralizing and non-neutralizing Fabs to gp120 inhibited by CD4. This implies that blocking of the CD4-gp120 interaction is unlikely to be an important factor in Fab neutralization of the HIV-1 virus.

Similar competition assays were performed with the Fabs panned against the four HIV-1 derived antigens. The 19 Fabs derived from panning against gp120 (IIIB) showed apparent affinities (1/concentration at 50% inhibition) for gp120 (IIIB) in the range $10^7 - 10^{-9}$ M with most being 1-3 X 10^{-8} M. The panning procedure tends to select strongly for tight binders so a grouping into a relatively narrow band of affinities was expected. Of 16 Fabs derived from panning against gp160 (IIIB), 6 were also reactive with gp120 (IIIB) and competition ELISAs showed they had similar apparent affinities as the gp120-panned Fabs. The non-gp120 reactive clones from the gp160 panning showed a lower ELISA reactivity with gp160 and could not be satisfactorily competed with gp160. They may be directed against gp41 but were not pursued here. Eight Fabs derived from panning against gp120 (SF2) also showed strong ELISA reactivity with gp120 (IIIB) and gave similar apparent binding affinities. Four Fabs were derived from panning against the V3 loop peptide. Of these Fabs, 2 reacted in ELISA with gp120 (SF2) but none with gp120 (IIIB). The apparent binding affinity of these loop binders to gp120 (SF2) was 10^{-8} M.

To complete the survey in terms of strain cross-reactivity of Fabs, those derived from the gp120 and gp160 (IIIB) pannings were examined for ELISA reactivity with gp120 (SF2). All were reactive.

Therefore, all the Fabs examined, with the exception of those selected by panning against the V3 loop peptide, bound to gp120 from IIIB and SF2 strains.

The Fabs were screened for CD4 inhibition of their binding to gp120 (IIIB) immobilized on ELISA wells. All, again with the exception of the V3 loop binders, showed sensitivity to CD4 inhibition. The inhibition constants were in the range 10^{-7} to 10^{-9} M.

c)   Binding Affinity Determination
        Using Surface Plasmon Resonance

Binding affinities were determined for six of the Fabs using surface plasmon resonance. Surface plasmon resonance was performed as it is a more accurate method for measuring affinity than competition ELISA. The six Fabs were chosen based upon sequence analysis which revealed that the heavy chains could be organized into 7 groups (Example 9). Each group contained members with identical V-D and D-J joining regions, implying a common clonal origin with varying numbers of differences elsewhere in the VH domain. Six Fabs were chosen as a representative of each respective group for further study as described herein. The single member of the seventh group was not included in these studies. The binding affinities of the six Fabs that are directed against the CD4 binding site of the gp120 envelope glycoprotein were determined using surface plasmon resonance as follows.

A Pharmacia BIAcore machine was used for the binding affinity determinations as previously described in Malmborg, et al., J. Immunol., 35:643-650 (1992) and Mattsson, et al., J. Immunol. Meth., 145:229-240 (1991). Optimization for the Fab fragments involved a number of steps. Two separate

channels on a biosensor chip were coated with gp120
derived from the HIV-1 strain LAI (Repligen, Cambridge
MA) such that one channel could be used for the
determination of on-rate constants (k_{on}) and the other

5   for the determination of off-rate constants (k_{off}).

For immobilization of antigen on the sensor
surfaces, a flow rate of 5 μl/min of PBS, pH 7.4 was
established over the biosensor chip. The chip was
then activated by injecting 30 μl of activation

10   solution (Pharmacia Biosensor, 50% 0.2 M N-ethyl-N'-
(3-diethylaminopropyl)-carbodiimide, 50% N-
hydroxysuccinimide). The flow rate was then adjusted
to 10 μl/min and the gp120 was injected in 10 mM
sodium acetate buffer, pH 4.5. When association rates

15   were to be determined, 25 μl of gp120 at 10 μg/ml was
injected (a final level of 4000 Response Units (RU)).
Twenty μl of gp120 at 2 μg/ml were injected for the
determination of dissociation constants (a final level
of 800 RU). In both cases, a flow rate of 5 μl/min

20   was reestablished following the gp120 injection and
the chip was blocked from any further immobilization
by the injection of 30 μl of 1 M ethanolamine, pH 8.5
(Pharmacia Biosensor).

For determination of on-rate constants (k_{on}), a

25   series of dilutions were made for each Fab to give
final concentrations in the range of 1 to 20 μg/ml.
30 μl of each Fab solution was injected in separate
experiments over the immobilized gp120 at a flow rate
of 5 μl/min. The change in response per unit time

30   (dR/dt) was plotted against time (t) for each
concentration. The slopes of each of these graphs
were then plotted against their corresponding
concentrations to give a final graph from which the
on-rate constant could be read.

35   For determination of off-rate constants (k_{off}), 30

μl of each Fab solution at 150 μg/ml were injected
over the immobilized antigen at a flow rate of 5
μl/min.   Once the reaction had reached equilibrium,
the Fab was removed from the antigen at a constant

5   flow rate of 50 μl/min.   A plot was then made of
$\ln(R_i/R_0)$ against t_i-t_0 for the dissociation phase. R_i
is the response at time t_i and R_0 is the initial
response at time t_0.   The slope of this graph was
taken to be the off-rate constant.   Affinities (K_a)

10   were then calculated and expressed as k_{on}/k_{off}.

The apparent affinities of the panel of
recombinant Fabs isolated from the donor as determined
in competition ELISA and surface plasmon resonance
were compared.   Values of approximately $10^8 M^{-1}$ were

15   obtained by competition ELISA as described in Example
2b6c in which the soluble and immobilized gp120
competed for binding to Fab in bacterial supernatants.
Such a methodology only gives an approximate measure
of affinity.   Therefore, the affinities of six of

20   these Fabs were measured using real-time biospecific
interaction analysis (surface plasmon resonance) in
order to obtain more accurate affinity constant
values.   The results are reproducible with a standard
deviation from the mean of approximately 5% as

25   determined by calculating a number of the affinity
constants in triplicate.   All Fabs examined have
affinities in the range of 5 x 10^7 to 1 x 10^8 M^{-1} as
determined in surface plasmon resonance (Table 4).
These values are in broad agreement with those derived

30   from competition ELISA.   These values imply no
correlation between affinity for recombinant gp120
derived from LAI and the ability to neutralize the
HXBc2 clone of HIV-1 derived from LAI as assessed in
Example 3c.

35

Table 4

Fab	k_{on} $(M^{-1}s^{-1})$	k_{off} (s^{-1})	$K_{a(M-1)}$
b3	9.6×10^3	1.8×10^{-4}	5.1×10^7
b6	1.6×10^4	1.6×10^{-4}	9.7×10^7
b11	5.6×10^4	4.3×10^{-4}	1.3×10^8
b12	4.5×10^4	4.3×10^{-4}	1.1×10^8
b13	1.1×10^4	1.4×10^{-4}	7.9×10^7
b14	6.0×10^4	6.5×10^{-4}	9.2×10^7

Also contemplated are competition ELISA and surface plasmon resonance assays where the binding of HIV-1 recombinant Fabs of this invention is performed in the presence of excess Fabs of this invention as well as those HIV-1 antibodies, polyclonal or monoclonal, present in patient sera, either asymptomatic or symptomatic, or obtained by other means such as EBV transformation and the like. The ability of an exogenously admixed antibody to compete for the binding of a characterized Fab of this invention will allow for the determination of equivalent antibodies in addition to unique epitopes and binding specificities.

3.   <u>Neutralizing Activity of Recombinant Human Fab Fragments Against HIV-1 In Vitro</u>

Binding of antibodies to viruses can result in loss of infectivity or neutralization and, although not the only defense mechanism against viruses, it is widely accepted that antibodies have an important role

to play. However, understanding of the molecular
principles underlying antibody neutralization is
limited and lags behind that of the other effector
functions of antibody. Such understanding is required

5   for the rational design of vaccines and for the most
effective use of passive antibody for prophylaxis or
therapy. This is particularly urgent for the human
immunodeficiency viruses.

A number of studies have led to the general

10   conclusion that viruses are neutralized by more than
one mechanism and the one employed will depend on
factors such as the nature of the virus, the epitope
recognized, the isotype of the antibody, the cell
receptor used for viral entry and the virus:antibody

15   ratio. The principle mechanisms of neutralization can
be considered as aggregation of virions, inhibition of
attachment of virus to cell receptor and inhibition of
events following attachment such as fusion of viral
and cellular membranes and secondary uncoating of the

20   virion. One of the important features of the third
mechanism is that it may require far less than the
approximately stoichiometric amounts of antibody
expected for the first two mechanisms since occupation
of a small number of critical sites on the virion may

25   be sufficient for neutralization. For instance it has
been shown that neutralization of the influenza A
virion obeys single hit kinetics as described by
Outlaw et al., <u>Epidemiol. Infect.</u>, 106:205-220 (1992).

Intensive studies have been carried out on

30   antibody neutralization of HIV-1. For review, see
Nara et al., <u>FASEB J.</u>, 5:2437-2455 (1991). Most have
focussed on a single linear epitope in the third
hypervariable domain of the viral envelope
glycoprotein gp120 known as the V3 loop. Antibodies

35   to this loop are suggested to neutralize by inhibiting

fusion of viral and cell membranes. Binding to the
loop resulting in neutralization can occur prior to
virus-cell interaction or following gp120 binding to
CD4. See, Nara, In Retroviruses of Human Aids and
5   Related Animal Diseases, eds. Girard et al., pp.
138-150 (1988); Linsely et al., J. Virol.,
62:3695-3702 (1988); and Skinner et al., J. Virol.,
67:4195-4200 (1988). Features of the V3 loop are
sequence variability within the loop [Goudsmit et al.,
10   FASEB J., 5:2427-2436 (1991) and Albert et al., AIDS,
4:107-112 (1990)] and sensitivity of neutralizing
antibodies against the loop to sequence variations
outside the loop [Nara et al., FASEB J., 5:2437-2455
(1991); Albert et al., supra; McKeating et al., AIDS,
15   3:777-784 (1989); and Wahlberg et al., AIDS Res. Hum.
Retroviruses, 7:983-990 (1991). Hence anti-V3 loop
antibodies are often strain specific and mutations in
the loop in vivo may provide a mechanism for viral
escape from antibody neutralization.

20   Recently considerable interest has focused on
antibodies capable of blocking CD4 binding to gp120.
A number of groups have described the features of
these antibodies as (a) reacting with conformational
i.e., non-linear epitopes, (b) reacting with a wide
25   range of virus isolates and (c) being the predominant
neutralizing antibodies in humans after longer periods
of infection. See, Berkower,et al., J. Virol.,
65:5983-5990 (1991); Steimer et al., Science,
254:105-108 (1991); Ho et al., J. Virol., 65:489-493
30   (1991); Kang et al., Proc. Natl. Acad. Sci., USA,
88:6171-6175 (1991); Posner et al., J. Immunol.,
146:4325-4332 (1991); and Tilley et al., Res. Virol.,
142:247-259 (1991). Neutralizing antibodies of this
type would appear to present a promising target for
35   potential therapeutics. The mechanism(s) of

neutralization of these antibodies is unknown although
there is some indication that this may not be blocking
of virus attachment since a number of mouse monoclonal
antibodies inhibiting CD4 binding to gp120 are either
5   non-neutralizing or only weakly neutralizing.

The generation of human monoclonal antibodies
against the envelope of HIV-1 as described by Burton
et al., Proc. Natl. Acad. Sci., USA, 88:10134-10137
(1991) using combinatorial libraries allows a novel
10   approach to the problem of neutralization. Given the
lack of a three-dimensional structure for gp120 and
the complexity of the virus, the approach seeks to
explore neutralization at the molecular level through
the behavior of related antibodies. This is possible
15   for the following reasons: (1) the combinatorial
approach allows the rapid generation of large numbers
of human antibodies; (2) the antibodies (Fab
fragments) are expressed in E.coli and can readily be
sequenced; and (3) antibodies have similar sequences
20   and common structural motifs allowing functional
differences to be meaningfully correlated with primary
structure.

Neutralization studies were performed as
described herein on the human recombinant Fab
25   fragments from 20 clones against gp120 prepared as
described in Examples 1 and 2, all of which are strain
cross-reactive and inhibited by CD4 from binding to
gp120. The results presented herein show that
neutralization was not effected by virus aggregation
30   or cross-linking of gp120 molecules on the virion
surface and was not correlated with blocking of the
interaction between soluble CD4 and recombinant gp120.

Neutralization studies were also performed as
described herein on the human recombinant Fab
35   fragments from the gp41-reactive clones prepared as

described in Examples 1 and 2.   The results are
presented below.

     Two different assays, a p24 ELISA assay and a
syncytium assay, were performed to measure
neutralization ability of the recombinant human HIV-1
immunoreactive Fabs.   An additional assay, a plaque
assay, was performed for determining the
neutralization effectiveness of the gp41-reactive
Fabs.   In plaque assays, CD4+ cells were cultured in
the presence or absence of soluble gp41-reactive Fabs
prior to inoculation with virus.   Inhibition of
infectivity, also referred to as neutralization, by
antibodies was expressed as the percent of plaque
formation in the cultures compared to cells exposed to
PBS alone.

     Neutralization assays were also performed with an
antibody molecule consisting of the light chain and
the VH region of the Fab 12 and the constant regions
(CH1, CH2, and CH3) of an IgG1 molecule.   Quantitative
infectivity microplaque and syncytial formation assays
to measure neutralization were performed with the b12
IgG1 and laboratory isolates MN and IIIB of HIV-1
virus.   In the syncytial formation assay, virus was
grown in H9 cells and infectivity measured by
culturing monolayers of CEM-SS target cells with 100-
200 syncytial forming units (SFUs) of virus, in the
presence or absence of antibody.   p24 ELISA and
microplaque formation assays were also performed with
primary clinical isolates of the HIV-1 virus.

     In addition, the ability of the recombinant human
HIV-1 immunoreactive Fabs b3, b6, b12, b13, and b12 to
neutralize the HXBc2 molecular clone of gp120 derived
from HTLV-IIIB (LAI) was determined in an envelope
complementation assay.   The supernatant containing
recombinant HIV-1 virions from cotransfected COS-1

cells was incubated with the recombinant Fabs prior to incubation with Jurkat cells. The recombinant HIV-1 virions contained the HXBc2 clone of HIV-1 strain LAI which encodes a chloramphenicol acetyltransferase (CAT) gene. Upon infection of Jurkat cells with the recombinant HIV-1 virions, the CAT gene was expressed and CAT activity measured. Activity of the CAT gene was therefore an indication of infectivity of the Jurkat cells with the recombinant HIV-1 virion. Lack of CAT activity indicated the Jurkat cells were not infected with the recombinant HIV-1 virion.

For some of these assays, the recombinant Fabs were first purified. One liter cultures of SB containing 50 μg/ml carbenicillin and 20 mM MgCl$_2$ were inoculated with appropriate clones and induced 7 hours later with 2 mM IPTG and grown overnight at 30°C. The cell pellets were sonicated and the resultant supernatant were concentrated to a 50 ml volume. The filtered supernatants were loaded on a 25 ml protein G-anti-Fab column, washed with 120 ml buffer at a rate of 3 ml/minute and eluted with citric acid at pH 2.3. The neutralized fractions were then concentrated and exchanged into 50 mM MES at pH 6.0 and loaded onto a 2 ml Mono-S column at a rate of 1 ml/minute. A gradient of 0-500 mM NaCl was run at 1 ml/minute with the Fab eluting in the range of 200-250 mM NaCl. After concentrating, the Fabs were positive when titered on ELISA against gp120 and gave a single band at 50 kD by 10-15% SDS-PAGE. Concentration was determined by absorbance measurement at 280nm using an extinction coefficient (1 mg/ml) of 1.4.

a.   Neutralization as Measured by the p24 ELISA
      Assay
      For this assay, diluted tissue culture

supernatants of HIV-1 IIIB or MN-infected peripheral
blood mononuclear cells (PBMC) (50TCID$_{50}$ (50% tissue
culture infectious dose), 100 μl) were maintained for
2 hours at 37°C with serial dilutions (1:2), beginning

5   at a dilution of 1:20, of recombinant Fab supernates
prepared in Example 2b6). Control Fab supernates were
also provided that included human neutralizing sera, a
known human neutralizing monoclonal antibody 2F5 and
the Fab fragment derived from that antibody by papain

10   digestion, and a known mouse neutralizing monoclonal
antibody and its F(ab')$_2$ fragment as described by
Broliden et al., J. Virol., 64:936-940 (1990). PBMC
(1 x 10^5 cells) were admixed to the virus/antibody
admixture and maintained for 1 hour at 37°C.

15   Thereafter, the cells were washed and maintained in
RPMI 1640 medium (GIBCO) supplemented with 10% fetal
calf serum, 1% glutamine, antibiotics and IL-2. The
culture medium was changed at days 1 and 4. At 7 days
post-infection, supernates were collected and analyzed

20   by HIV-1 p24 antigen capture ELISA as described by
Sundqvist et al., J. Med. Virol., 29:170-175 (1989)
the disclosure of which is hereby incorporated by
reference. Neutralization was defined as positive if
an 80% or greater reduction of optical density at

25   490nm in the culture supernatant occurred as compared
to negative Fab or negative human serum. Tests with
all Fabs, mAbs and sera were repeated on at least two
occasions.

30   b.   Quantitative Infectivity Assay Based on
                  Syncytial Formation
            A quantitative neutralization assay with the
MN strain of HIV-1 was performed as described by Nara
et al., AIDS Res. Human Retroviruses, 3:283-302

35   (1987), the disclosure of which is hereby incorporated

by reference. Monolayers of CEM-SS target cells were
cultured with virus, in the presence or absence of
antibody, and the number of syncytia forming units
determined 3-5 days later. An equivalent amount of
virus was used in the assays to allow direct
comparison of the various antibody concentrations
tested. The assays were repeatable over a virus-
surviving fraction range of 1 to 0.001 within a 2 to
4-fold difference in the concentration of antibody
(P<0.001).

      c.   Neutralization as Measured by the Envelope
            Complementation Assay

           The ability of purified recombinant Fabs b3,
b6, b11, b12, b13, and b14 to neutralize the HXBc2
gp120 molecular clone of the HIV-1 (LAI) isolate was
assessed in an envelope complementation assay (Helseth
et al., J. Virol., 65:2119-2123 (1991)). Briefly,
COS-1 cells were cotransfected with a plasmid
expressing envelope glycoprotein 120 derived from HIV-
1 (LAI) and a plasmid containing an env-defective
HXBc2 clone and encoding the bacterial CAT gene.
Equal fractions of the cell supernatants containing
recombinant virions were incubated at 37°C for 1 hour
with varying concentrations of recombinant Fab (0.1 -
20 μg/ml) or control monoclonal antibody 110.4 prior
to incubation with Jurkat cells. Three days post-
infection, the Jurkat cells were lysed and CAT
activity measured. Neutralization was expressed as a
decrease in the percentage of residual chloramphenicol
transferase (CAT) activity. Control monoclonal
antibody 110.4 is a strongly neutralizing antibody
directed to the V3 loop of the HXBc2 HIV-1 strain.

      d.   Results of the Neutralization Assays for

gp120

Assays were generally repeated at least
twice with reproducible results. For the data
reported in Figure 6, the gp120-specific Fab
supernates were divided into two parts, one being used
in the p24 assay and the other in the syncytia assay.
A dash (-) indicates that there was no neutralization
at 1:20 dilution in the p24 assay and 1:16 in the
syncytial assay (with most clones showing no
detectable neutralization at a 1:4 dilution).
Neutralization titers are indicated in the figure.
For the p24 assay, the titer corresponds to the
greatest dilution producing >80% reduction in
absorbance in ELISA. For the syncytia assay, Fabs 4
and 12 produced >95% neutralization at a 1:4 dilution
of supernate and 80 and 70% reduction at 1:128
dilution respectively. These Fabs were effective
neutralizers in both types of assays. They have also
been shown to neutralize infection by IIIB and RF
strains using a PCR-based assay of proviral
integration. Fabs 6 and 7 showed no neutralization in
the syncytia assay but other supernate preparations
showed activity. Fab 13 was consistently effective in
the p24 assay but not in the syncytia assay. A number
of other clones show lower levels of neutralizing
ability.

Fabs were purified from a selection of some of
the clones as described above and used in both
neutralization assays. As shown in Figure 9, Fabs 4
and 12 were again effective in both assays at similar
levels with for example 50% inhibition of syncytial
formation at an Fab concentration of approximately 20
nM (1 μg/ml). The results shown are derived from the
syncytia assay using the MN strain. Fabs 7 and 21
were equally effective in the syncytial assay but

somewhat less so in the p24 assay. The p24 assay
indicated greater than 80% neutralization of HIV-1 MN
strain for Fab 4 at 3, Fab 7 at 15, Fab 12 at 3, Fab
13 at 4 and Fab 21 at 7 μg/ml, respectively. Fab 13

5   however was ineffective in the syncytial assay at 25
μg/ml. For the IIIB strain, greater than 80%
neutralization was observed for Fab 4 at 13, Fab 7 at
15, Fab 12 at 7 and Fab 21 at 14 μg/ml, respectively.
Although Fab 11 was not effective in neutralization

10   assays when unpurified as shown in Figure 6, following
purification, Fab 11 was equally effective as Fab 12
in neutralizing HIV-1. For this reason, the Fab is
being deposited with the ATCC as described in Example
12 along with Fab 12 and Fab 13.

15   The ability of purified recombinant Fabs b3, b6,
b11, b12, b13, and b14 to neutralize the HXBc2 gp120
molecular clone of the HIV-1 (LAI) isolate was
assessed in an envelope complementation assay. Figure
23 shows the concentration dependence of Fab

20   neutralization of the HXBc2 clone in this assay. All
of the Fabs neutralize effectively at the highest
concentration measured (20 μg/ml). Irrelevant Fabs,
Fabs directed to surface glycoproteins on other
viruses such as RSV, do not neutralize in this assay.

25   Examination of the lower concentrations clearly
reveals that Fab b12 is the most effective
neutralizer. The neutralizing potency of Fab b12 was
greater than that of the 110.4 whole monoclonal
antibody tested in parallel. The 110.4 antibody is

30   one of the most potent antibodies directed against the
V3 loop of the HXBc2 HIV-1 strain (Thali, M. and J.
Sodroski, unpublished observations). In other
studies, Fab b12 has been found to show exceptional
neutralizing ability towards laboratory (Example 3 and

35   Barbas et al., Proc. Natl. Acad. Sci., USA, 91, in

press (1994)) and field isolates of HIV-1 as described
in Example 5.

There are a number of conclusions arising from
the data shown in the Figures 6, 9 and 23. It is
5   apparent that HIV-1 can be neutralized without virion
aggregation or cross-linking of gp120 molecules on the
virion surface since monovalent Fab fragments are
effective. To further confirm this finding, a Fab
fragment was produced by papain digestion of a known
10   neutralizing human monoclonal antibody. As shown in
Figure 6, the Fab fragment was approximately equally
effective as the whole IgG in neutralization of the MN
strain of HIV-1. This is consistent with results on
Fabs prepared from two mouse monoclonal antibodies to
15   the V3 loop. An F(ab')$_2$ fragment of a mouse
monoclonal antibody was somewhat less effective than
the parent IgG in neutralization of the MN strain.
Interestingly, the fragments from these control
antibodies were relatively poor in neutralizing the
20   IIIB strain of HIV-1. The results also show that
there appears to be a difference between the two
assays employed since Fab 13 was consistently
effective in one assay but not the other. The
principal variables were the incubation time of the
25   virus and antibody prior to infection (2 hours for the
p24 assay and 0.5 hours for the syncytial assay), the
amount of virus used for infection, the cells used to
propagate virus (human PBMCs for the former and H9
cells for the latter) and the cells infected (human
30   PBMCs for the former and CEM.SS cells for the latter).
Of these, there is a strong possibility that the MN
virus used in the two assays, having been passaged
through different cells, is critically different.

The Fabs show a spectrum of neutralizing ability
35   for gp120 from a molecular clone HXBc2 derived from

the HIV-1 strain LAI in the envelope complementation
assay. Fab b12 exhibited the greatest potency of
neutralization and was even more effective in this
assay than a whole antibody directed to the V3 loop of
gp120. Neutralizing ability is not correlated with
either the apparent affinity of the Fab for gp120
derived from the recombinant HIV-1 strain LAI as
estimated by competition ELISA or the affinity for
gp120 derived from HIV-1 strain LAI as determined by
surface plasmon resonance. For example, Fabs b6, b12,
and b14 have very similar affinities by surface
plasmon resonance (Table 4) but different
neutralization ability in the envelope complementation
assay (Figure 23). Similarly, neutralization is not
correlated with the ability of the Fab to compete with
soluble CD4 in a competition ELISA.

    e.   <u>Results of the Neutralization Assays for</u>
        <u>gp41</u>

    The gp41-reactive Fabs exhibited specificity
to the conformation epitope of gp41 including amino
acid residues in positions 565-585 and 644-663. The
five selected gp41-specific Fabs were designated DL 41
19, DO 41 11, GL 41 1, MT 41 12 and SS 41 8.
Neutralization assays were performed as described
above for the gp120-reactive Fabs. In the plaque
assays, the data shown is the concentration of Fab in
micrograms/milliliter required to achieve 50% of
neutralization. The data for the other two
neutralization assays is also expressed in
micrograms/milliliter of Fab required to neutralize
infection as defined in the description of the p24 and
syncytial assays above. The results of the three
neutralization assays, plaque, syncytial and p24, are
presented in Table 5. The MN and IIIB HIV strains

were used as indicated in Table 5 for the assays. The
abbreviation "ND" stands for not determined when
indicated in the table.

5

Table 5
Assay/Strain

Fab	MN	Plaque IIIB	Syncytial IIIB	P24 MN	IIIB
DL 41 19	<4	<40	1.4	ND	ND
DO 41 11	<40	7.1	2.3	0.9	ND
GL 41 1	<4	<4	1.7	ND	3.5
MT 41 12	<40	<40	5.5	4.5	4.5
SS 41 8	<4	<4	2.2	ND	7.1

15

As shown in Table 5, all five Fabs were effective
at neutralizing both MN and IIIB strains of HIV in
either plaque, syncytial or p24 assays. Fabs DL 41 19
and DO 41 11 exhibited strain specificity in the
plaque assay where the former was ten-fold more
effective at inhibiting plaque formation with the MN
strain than with the IIIB strain. The opposite
specificity was seen with the DO 41 11 Fab. However,
both Fabs exhibited comparable neutralization as
measured by the syncytial assay. Two Fabs, GL 41 1
and SS 41 8, were equally effective at inhibiting
plaque formation with either MN or IIIB strains. The
Fab MT 41 12 was similarly not strain-specific
although neutralization required 10 fold more
antibody. No strain specificity was evident when Fab
MT 41 12 was used in p24 assays where the same amount
of antibody was equally effective. All five
antibodies were neutralized IIIB as measured in the
syncytial assay.

Thus, the five gp41-specific Fabs neutralized

HIV-1 MN and IIIB in at least two of the three assays used for measuring neutralizing activity. Moreover, strain specificity was prevalent in two of the five assays as measured by the plaque assay. Based on these differential neutralization characteristics, the gp41-specific Fabs provide useful therapeutic reagents for neutralizing HIV-1.

4.   Construction of a Mammalian Expression Vector pEe12 Combo BM 12 for the Expression of an IgG1 Antibody Molecule with the Fab from b12 (b12 IgG1)

Although Fab b12 is capable of neutralizing some primary isolates, the corresponding whole antibody molecule is likely to be more effective. The whole antibody, consisting of the Fab fragment and the Fc domain, participates in the elimination of foreign cells by first binding specifically to the foreign cell via the Fab portion and interacting with other cells in the immune system via the Fc domain. The Fc domain also enables the antibody to bind complement.

Fab b12 was converted to a whole IgG1 molecule (b12 IgG1) by cassetting the variable heavy chain (VH) and light chain genes into a vector created for high-level mammalian expression. b12 IgG1 used in the neutralization studies was prepared by expression in Chinese hamster ovary (CHO) cells and purified by affinity chromatography.

The strategy to convert the Fab b12 to a whole IgG1 molecule was similar to that described previously for the generation of a whole antibody beginning with a phage derived Fab (Bender, et al., Hum. Antibod. Hybridomas, 4:74-79 (1992)).

a.   Construction of b12 Heavy Chain IgG1 pSG-5
      Mammalian Expression Vector

1)   Modification of b12 Heavy Chain
      Variable Region to Introduce a Kozak
      Sequence, Mammalian Leader Sequence,
      and Human VH Consensus Sequence

              First, the b12 VH region was cloned
into a pSG-5 expression vector (Green et al., Nucl.
Acids Res., 16:369 (1988)) to fuse the b12 VH to the
heavy chain constant domains (CH1, CH2, and CH3) of an
IgG1 antibody molecule. The double-stranded Fab b12
DNA was used as a template for isolating the gene
encoding the VH region of the Fab b12, the amino acid
residue sequence of which is listed in SEQ ID NO
66.   Fab b12 DNA and mouse B73.2 IgG1 DNA
(Whittle, et al., Protein Eng., 1:499 (1987) and
Bruggmeman, et al., J. Exp. Med., 166:1351 (1987))
were used as templates for a PCR amplification for the
construction of a DNA fragment consisting of the
unique Kozak sequence for the control of heavy chain
expression, the mouse B72.3 heavy chain leader
sequence (MEWSWVFLFFLSVTTGVHS), the human VH consensus
sequence (QVQLVQ), and the VH region of the Fab b12.
Altering the beginning of the VH from the mouse
consensus sequence to the human consensus sequence
also destroyed the original Xho I cloning site. The
restriction sites EcoR I and Sst I were introduced in
the amplification reaction and were located at the 5'
and 3' ends of the fragment, respectively. The
procedure for creating the modified VH fragment by
combining the products of the two separate PCR
amplifications is described below.

              The primer pair, HC-1 (SEQ ID NO 141) and HC-2
(SEQ ID NO 142) as shown in Table 10, was used in the

first PCR reaction to amplify a portion of the Fab b12
VH gene and incorporate the human heavy chain
consensus sequence into the 5' end of the VH fragment
and introduce an Sst I cloning site in the 3' end of

5   the VH fragment. In addition, the 5' PCR primer
introduces sequences into the VH fragment which form
27 base pairs of homology with the mouse leader
sequence fragment prepared below. The 27 base pairs
of homology in the fragments is used in a subsequent

10   PCR reaction to fuse the two PCR products (Yon and
Fried, Nucl. Acids Res., 17:4895 (1989)) to form a
modified VH fragment consisting of the EcoR I cloning
site, the mouse leader sequence 72.3, the human
consensus sequence, the remaining VH coding sequence,

15   and the Sst I cloning site. For the PCR reactions, 1
μl containing 100 ng of Fab b12 DNA was admixed with
10 μl of 10X PCR buffer in a 0.5 ml microfuge tube.
To the DNA admixture, 8 μl of a 2.5 mM solution of
dNTPs (dATP, dCTP, dGTP, dTTP) was admixed to result

20   in a final concentration of 200 micromolar (μM) of
each dNTP. 1 μl (equivalent to 20 picomoles (pM)) of
the 5' forward HC-1 primer and 1 μl (20 pM) of the 3'
backward HC-2 primer were admixed into the DNA
solution. To the admixture, 73 μl of sterile water

25   and 2.5 units of Taq DNA polymerase was added. Two
drops of mineral oil were placed on top of the
admixture and 35 rounds of PCR amplification in a
thermocycler were performed. The amplification cycle
consisted of 52°C for 1 minute, 72°C for 2 minutes and

30   94°C for 0.5 minutes.
     The primer pair, HC-3 (SEQ ID NO 143) and HC-4
(SEQ ID NO 144) as shown in Table 10, was used in a
separate PCR reaction to amplify the mouse B72.3
leader sequence and incorporate an EcoR I cloning site

35   at the 5' end of the fragment and to introduce a 27

base pair sequence which has homology to the modified
VH fragment prepared above. Double-stranded DNA
encoding the mouse B73.2 IgG1 (Whittle, et al., _supra_)
was used as a template for preparation of the mouse

5   72.3 leader sequence. The PCR reaction to prepare the
mouse leader sequence fragment was performed using the
same conditions as described above for the preparation
of the modified VH fragment.

The resultant PCR modified b12 VH DNA fragment

10   and mouse leader sequence fragment were purified by
electrophoresis in a 2.5% Nu-Sieve agarose gel (FMC).
The area in the agarose containing the modified b12 VH
DNA fragment and mouse leader sequence fragment were
excised from the agarose.

15   A third PCR amplification using the primer pairs,
HC-1 (SEQ ID NO 141) and HC-3 (SEQ ID NO 143) as shown
in Table 10, was performed to fuse the mouse leader
fragment with the modified VH fragment. The primers
used for this amplification were designed to preserve

20   an EcoR I site, a unique Kozak sequence, and the mouse
B72.3 heavy chain leader sequence on the 5' end of the
amplified fragment and to preserve the Sst I cloning
site on the 5' end of the amplified fragment. The
templates used in this PCR reaction were the two

25   purified PCR reaction products described above. The
PCR reaction and subsequent purification of the PCR
product were performed as described above.

2)   <u>Modification of b12 Heavy Chain</u>
30   <u>Variable Region to Eliminate a BglII</u>
<u>Restriction Site</u>

The b12 modified heavy chain fragment
prepared in Example 4a1 contained a Bgl II cloning
site at amino acid residue 87 which would interfere

35   with the insertion of the heavy chain fragment into

the pEE6 mammalian expression vector. The Bgl II
restriction site was therefore eliminated in a PCR
reaction using primers which destroyed the Bgl II
restriction site while preserving the encoded amino
acid, arginine at amino acid residue 87 of the
modified b12 heavy chain fragment.

The primer pair, HC-1 (SEQ ID NO 141) and HC-6
(SEQ ID NO 146) as shown in Table 10, was used in the
first PCR reaction to preserve the 5' region of the
modified b12 heavy chain fragment and destroy the Bgl
II restriction site at amino acid residue 87 of the
heavy chain. The HC-6 primer introduces sequences
into the VH fragment which form 32 base pairs of
homology with the remaining portion of the VH fragment
which will be prepared as described below. The 32
base pairs of homology in the fragments was used in a
subsequent PCR reaction to fuse the two PCR products
(Yon and Fried, supra) to form a modified VH fragment
as described above but without the Bgl II restriction
site. The PCR reaction was performed and the PCR
products were purified as described in Example 4a1.

The primer pair, HC-2 (SEQ ID NO 142) and HC-5
(SEQ ID NO 145) as shown in Table 10, was used in the
second PCR reaction to preserve the 3' region of the
modified b12 heavy chain fragment and destroy the Bgl
II restriction site. The HC-5 primer introduces
sequences into the VH fragment which form 32 base
pairs of homology with the remaining portion of the VH
fragment which was prepared in the first PCR reaction.
PCR products which have incorporated the HC-5 and HC-6
primers contain 32 base pairs of overlapping sequences
which are identical. It is the annealing of the two
PCR products at these 32 base pairs during the
subsequent PCR reaction which fuses the two portions
of the VH fragment together to recreate the entire VH

fragment as described in Yon and Fried (supra).

A third PCR amplification using the primer pairs, HC-1 (SEQ ID NO 141) and HC-3 (SEQ ID NO 143) as shown in Table 10, was performed to fuse the two VH

5 fragments in which the Bgl II restriction site had been destroyed. The primers used for this amplification were designed to preserve an EcoR I site, a unique Kozak sequence, and the mouse B72.3 heavy chain leader sequence on the 5' end of the

10 amplified fragment and the Sst I cloning site on the 3' end of the amplified fragment. The templates used in this PCR reaction were the two purified PCR reaction products described above. The PCR reaction and subsequent purification of the PCR product were

15 performed as described in Example 4a1.

Table 10

SEQ

ID NO	Primer		
(141)[1] HC-1	(F)	5'	CAGGTTCAGCTGGTTCAGTCCGGGG CT 3'
(142)[2] HC-2	(B)	5'	CCTTGGAGCTCACGATGACCGTGGT TCCTTGGCCCCAGACGTCC3'
(143)[3] HC-3	(F)	5'	GGCCGCGAATTCGCCGCCACCATGG AATGGAGCTGGGTCTTTCTCTTCTT CCTGTCAGTA 3'
(144)[2] HC-4	(B)	5'	AGCCCCGGACTGAACCAGCTGAAC CTG 3'
(145)[4] HC-5	(F)	5'	GGAGTTGAGGAGCCTCAGGTCTGCA GACACGG 3'
(146)[4] HC-6	(B)	5'	CCGTGTCTGCAGACCTGTGGCTCCT CAACTCC 3'
(147) LC-1	(F)	5'	GATGCCAGATGTGAGATCGTTCTCA CGCAGTCT 3'
(148)[3,5] LC-2	(B)	5'	GCGGGATCCGAATTCTCTAGAATTA ACACTCTCCCCTGTTGAAGCTCTTT GTGACGGGCGAACTCAG 3'
(149)[3] LC-3	(F)	5'	GCGCGAATTCACCATGGGTGTGCCC ACTCAGGTCCTGGGGTTGCTGCTGC 3'
(150) LC-4	(B)	5'	AGACTGCGTGAGAACGATCTCACAT CTGGCATC 3'

(151)[6]   LC-5   (F)   5' GCGCAAGCTTACCATGGGTGTGCCC
                                ACTCAGGTCCTGGGGTTGCTGCTGC
                                3'

F   Forward Primer

B   Backward Primer

[1]   the Sst I cloning site is single underlined

[2]   the primers, HC-2 and HC-4 contain
complementary sequences

[3]   the EcoR I cloning site is single underlined

[4]   in HC-4, the G that is double underlined was
      altered from an A to eliminate a Bgl II restriction
      site; in HC-5, the C that is doubleunderlined was
      altered from a T to eliminate a Bgl II restriction
      site

[5]   the base A that is double underlined was introduced
      in the PCR primer to alter the encoded amino acid
from an arginine, R, to a serine, S

[6]   the HindIII cloning site is single underlined

        3)   Insertion of Modified b12 Heavy Chain
              Variable Region into the pSG-5
              Mammalian Expression Vector
              The modified b12 heavy chain variable
region PCR product was ligated into a mammalian
expression vector (Adair, et al., Hum. Antibod.
Hybridomas, in press). The mammalian expression
vector consisted of the pSG-5 vector (Figure 24) with
a human IgG1 gene inserted at the EcoR I site. The
human IgG1 gene contained a VH insert in the same
reading frame as the constant regions of the human
IgG1 gene. The VH insert was removed by digestion
with EcoR I and Sst I enzymes. The constant regions

(CH1, CH2, and CH3) remained in the pSG-5 vector. Transcription of the heavy chain gene in the pSG-5 expression vector is under the control of the SV40 early promoter. Transcriptional termination is signaled by the SV40 polyadenylation signal sequence downstream of the heavy chain sequence. The M13 intergenic region allows for the production of single-stranded DNA for nucleotide sequence determination.

The modified b12 heavy chain variable region PCR product was digested with EcoR I and Sst I and purified on a 2.5% Nu-Sieve agarose gel (FMC). The mammalian expression vector DNA containing the IgG1 sequences was digested in parallel with EcoR I and Sst I enzymes to remove the original VH region. The PCR modified heavy chain variable region was ligated to the constant regions in the mammalian expression vector using T4 DNA ligase under conditions well known to those of skill in the art and transformed into DH5α competent cells following the manufacturer's recommended procedures (GIBCO, BRL Life Technologies, Gaithersburg, MD). The PCR modified heavy chain variable region was inserted in the same reading frame as the constant regions of the human IgG1 gene in the pSG-5 vector. Miniprep DNAs were analyzed and large scale plasmid preparations performed. The nucleotide sequence of the 5' untranslated region including the Kozak sequence, mouse B72.3 heavy chain leader sequence, heavy chain variable region, heavy chain constant regions, and SV40 signal sequence was determined by the dideoxy-nucleotide chain termination method (Sanger et al., _supra_).

b.   Construction of a b12 Light Chain pSG-5
      Mammalian Expression Vector
      1)   Modification of b12 Light Chain to
            Introduce a Kozak Sequence, Mammalian
            Leader Sequence, and Human Light Chain
            Consensus Sequence

            The b12 light chain was cloned into a
separate pSG-5 expression vector (Green et al.,
supra). The double-stranded Fab b12 DNA was used as a
template for isolating the gene encoding the light
chain of the Fab b12, the amino acid residue sequence
the light chain of Fab b12 is listed in SEQ ID NO 97.
Mouse B73.2 IgG1 DNA (Whittle, et al., Protein Eng.,
1:499 (1987) and Bruggmeman, et al., J. Exp. Med.,
166:1351 (1987)) was used as a template for isolating
the mouse B73.2 leader sequence. Fab b12 and mouse
B73.2 IgG1 DNA were thus used as templates for a PCR
amplification for the construction of a DNA fragment
consisting of the unique Kozak sequence for control of
light chain expression, the mouse B72.3 light chain
leader sequence (MGVPTQLGLLLWLTDARC), and the b12
light chain beginning with a human light chain amino
acid consensus sequence (EIVLTQSP). Altering the
beginning of the light chain from the mouse amino acid
consensus sequence to the human amino acid consensus
sequence also destroys the original Sac I cloning
site. The restriction site, EcoR I, was introduced in
the amplification reactions and was located at both
the 5' and 3' ends of the fragment. The procedure for
creating this fragment by combining the products of
two separate PCR amplifications is described below.

            The primer pair, LC-1 (SEQ ID NO 147) and LC-2
(SEQ ID NO 148), was used in the first PCR reaction as
performed above to amplify the Fab b12 light chain
gene and incorporate the human light chain consensus

sequence into the fragment and the EcoR I cloning site
into the 3' end of the b12 light chain fragment. For
the PCR reaction, 1 μl containing 100 ng of Fab b12
DNA was admixed with 10 μl of 10X PCR buffer in a 0.5

5   ml microfuge tube. To the DNA admixture, 8 μl of a
2.5 mM solution of dNTPs (dATP, dCTP, dGTP, dTTP) was
admixed to result in a final concentration of 200 μM
of each dNTP. 1 μl (equivalent to 20 pM) of the LC-1
primer and 1 μl (20 pM) of the 3' backward LC-2 primer

10   was admixed into the DNA solution. To the admixture,
73 μl of sterile water and 2.5 units of Taq DNA
polymerase was added. Two drops of mineral oil were
placed on top of the admixture and 35 rounds of PCR
amplification in a thermocycler were performed. The

15   amplification cycle consisted of 52°C for 1 minute,
72°C for 2 minutes and 94°C for 0.5 minutes.

The primer pair, LC-3 (SEQ ID NO 149) and LC-4
(SEQ ID NO 150) as shown in Table 10, was used in a
separate PCR reaction to amplify the mouse light chain

20   B72.3 leader sequence and incorporate an EcoR I
cloning site at the 5' end of the fragment and to
introduce a 27 base pair sequence which has homology
to the modified light chain fragment prepared above.
Double-stranded DNA encoding the mouse B73.2 IgG1

25   (Whittle, et al., _supra_) was used as a template for
preparation of the mouse 72.3 leader sequence. The
PCR reaction to prepare the mouse leader sequence
fragment was performed using the same conditions as
described in Example 4a for the preparation of the

30   modified VH fragment.

The resultant PCR modified b12 light chain DNA
fragment and light chain mouse leader sequence
fragment were purified by electrophoresis in a 2.5%
Nu-Sieve agarose gel (FMC). The area in the agarose

35   containing the modified b12 light chain DNA fragment

and light chain mouse leader sequence fragment were
excised from the agarose.

A third PCR amplification using the primer pairs,
LC-1 (SEQ ID NO 141) and LC-4 (SEQ ID NO 150) as shown
in Table 10, was performed to fuse the light chain
mouse leader fragment with the modified light chain
fragment. The primers used for this amplification
were designed to preserve an EcoR I site, a unique
Kozak sequence, and the mouse B72.3 light chain leader
sequence on the 5' end of the amplified fragment and
to preserve the EcoR I cloning site on the 5' end of
the amplified fragment. The templates used in this
PCR reaction were the two purified PCR reaction
products described above. The PCR reaction and
subsequent purification of the PCR product were
performed as described in Example 4a1.

2)   Insertion of Modified b12 Light Chain
into pSG-5 Mammalian Expression Vector

The modified b12 light chain PCR
product was ligated to a pSG-5 vector (Figure 24).
The pSG-5 vector had the same features described in
Example 4a2 but did not contain a human IgG1 gene.

The modified b12 light chain PCR product was
digested with EcoR I and purified on a 2.5% Nu-Sieve
agarose gel (FMC). The pSG-5 vector DNA was digested
in parallel with EcoR I enzyme. The PCR modified
light chain was ligated to the pSG-5 vector using T4
DNA ligase (New England Biolabs, Beverly, MA) and
transformed into DH5α competent cells (GIBCO, BRL Life
Technologies, Gaithersburg, MD) following
manufacturer's instructions. Miniprep DNAs were
analyzed and isolation of plasmid DNA performed. The
nucleotide sequence of the light chain gene was
determined using the dideoxy-nucleotide chain

termination method (Sanger et al., _supra_). The
nucleotide sequence of the 5' untranslated region,
mouse B72.3 light chain leader sequence, light chain
variable region, light chain constant region, and SV40

5   signal sequence was obtained. The nucleotide and
amino acid residue sequences are illustrated in
Figures 25A and 25B and are given in the sequence
listing as SEQ ID NOs 152 and 153.

10   c.   Transient Expression of b12 Heavy and Light
              Chain Genes in pSG-5 Vectors in COS-7 Cells
          1)   Transient Expression of b12 IgG1 in
                COS-7 Cells
                The human heavy and light chains in

15   the separate pSG-5 expression vectors were
cotransformed and transiently expressed in COS-7
cells. COS-7 cells (SV40 transformed African Green
Monkey Kidney Cells) provide a rapid and convenient
method to test the expression and function of the

20   antibody genes. The COS-7 cells constituitively
express the SV40 large T antigen which supports the
transient replication of episomes carrying the SV40
origin of replication. The pSG-5 expression vector
has an SV40 origin of replication. Upon transfection

25   into COS-7 cells, the expression vectors are
replicated in the nucleus to a high copy number,
resulting in relatively high transient expression
levels.
                COS-7 cells were obtained from the American Type

30   Culture Collection (CRL 1651) and cultured in
Dulbecco's modified Eagle's medium (DMEM),
supplemented with 10% fetal bovine serum (GIBCO BRL,
Gaithersburg, MD) and 1% penicillin, and 1%
streptomycin. Transfections were performed with 10 μg

35   of plasmid DNA per 100 mm tissue culture plate

containing 1 x 10^6 cells. The control plate was
transfected with plasmid vector DNA without an insert.
The plates were incubated at 37°C after transfection.
The supernatants were harvested at 48 hours and tested

5   for gp120 binding specificity in an ELISA assay.

2)   ELISA Assay for the Detection of
Binding of b12 IgG1 to gp120
Supernatants from COS-7

10   transformants were tested for binding to gp120 in an
ELISA assay. Briefly, the ELISA plate was coated with
recombinant IIIB gp120 antigen at a concentration of 1
μg/ml. The serially diluted supernatant containing
the b12 antibody was added to the wells and incubated

15   at 37°C for 1 hour. After washing the plate to remove
unbound antibody, a goat anti-human Ig Fc horse radish
peroxidase (HRP) conjugated secondary antibody was
added and incubated for an additional hour. An OPD
substrate for the HRP conjugated antibody was added

20   and the HRP activity detected by determining the
absorbance at 490 nm.

d.   Insertion of the b12 Heavy Chain IgG1 into
the pEE6 Mammalian Expression Vector to

25   Create pEe6HC BM 12
After confirmation that the antibody
molecule expressed by the heavy and light chain pSG-5
expression vectors bound gp120 as described in Example
4c, the heavy chain was removed from the pSG-5 vector

30   and ligated into the pEE6 mammalian expression vector
(Bebbington et al., Bio/Technology, 10:169 (1992)).
The pEE6 vector (Celltech, England) contains an HCMV
promoter and the glutamine synthetase gene (GS). The
pEE6 vector was chosen because of the GS gene which

35   serves as a selectable marker. CHO cells are devoid

of GS activity and thus are dependent on a supply of
glutamine in the culture medium. Cells transfected
with the pEE6 vector containing the GS gene are able
to synthesize glutamine from glutamate and can survive
in the absence of glutamine in the culture medium.
For CHO cells, the addition of methyl sulfoxamine
(MSX) leads to amplification of the transfected
plasmid DNA.

The heavy chain pSG-5 vector was digested with
EcoR I and Bgl II to remove the 5' untranslated region
including the unique Kozak sequence, mouse heavy chain
B72.3 leader sequence, and heavy chain variable and
constant regions from the pSG-5 vector. The pEE6
vector was also digested with EcoR I and BamH I. Both
the vector and heavy chain DNAs were analyzed on a
0.7% low melting point agarose (LMPA) gel. The 3.5 kb
heavy chain band and the 4.68 kb pEE6 vector band were
excised from the gel and ligated together in the
presence of the LMPA at 15°C overnight with 1 μl of T4
DNA ligase and 1 μl of 10X ligase buffer (New England
Biolabs, Beverly, MA). Upon ligation, the EcoR I site
is reconstituted but the BamH I and BglII sites are
destroyed. Prior to transformation, 5 μl of the
ligated DNA in LMPA was diluted with 20 μl of TCM
buffer (10 mM tris, 10 mM $CaCl_2$, and 10 mM $MgCl_2$).
Only 10 μl of the 25 μl was used for the
transformation. The ligated circular plasmid DNA
construct was transformed into maximum efficiency DH5α
competent cells. The standard protocol for
transformation was used, wherein the DNA and 100 μl of
the competent bacterial mix (GIBCO BRL, Gaithersburg,
MA) were incubated on ice for 20 minutes and heat
shocked at 42°C followed by incubation on ice for 2
minutes. About 900 μl of SOC (GIBCO BRL,
Gaithersburg, MA) was added to the transformation.

Only 100 μl of the 1000 μl of the transformed cells
was plated on LB with carbenicillin plates
(carbenicillin at 50 μg/ml). The plates were
incubated at 37°C overnight. Twelve individual

5   colonies were picked for miniprep analysis. Several
diagnostic digests confirmed the presence of the heavy
chain insert. Plasmid DNA was isolated on a CsCl
gradient (Sambrook et al., supra). The nucleotide and
amino acid residue sequences are illustrated in

10   Figures 27A through 27E and the nucleotide and amino
acid residue sequences are given in the sequence
listing as SEQ ID NOs 154 and 155.

       e.   Insertion of the b12 Light Chain into the

15   pEE12 Mammalian Expression Vector
              The light chain was ligated into the pEE12
vector (Celltech, England) from the pSG-5 vector
involving similar steps as described in Example 4d for
the heavy chain. The pEE12 vector has a human CMV

20   promoter for expression of the light chain, a
polylinker to provide cloning sites, and a
polyadenylation signal for termination of
transcription. The vector also contains the GS
selectable marker gene, whose expression is controlled

25   by an SV40 early promoter at the 5' end of the GS
gene, an intron, and a polyadenylation signal at the
3' end of the GS gene.

       1)   Preparation of Modified b12 Light

30   Chain
              The 5' PCR primer was designed to
replace the EcoR I cloning site with a HindIII cloning
site. The 3' PCR primer maintained the EcoR I cloning
site.

35   The primer pair, LC-5 (SEQ ID NO 151) and LC-2

(SEQ ID NO 149), was used in the PCR reaction as described in Example 4a1 to amplify the Fab b12 light chain gene and incorporate HindIII and EcoR I cloning sites into 5' and 3' ends of the fragment,

5   respectively. The b12 pSG-5 vector containing the b12 light chain was used as the template in the PCR reaction. For the PCR reaction, 1 μl containing 100 ng of b12 pSG-5 DNA was admixed with 10 μl of 10X PCR buffer in a 0.5 ml microfuge tube. To the DNA

10   admixture, 8 μl of a 2.5 mM solution of dNTPs (dATP, dCTP, dGTP, dTTP) was admixed to result in a final concentration of 200 micromolar (μM) of each dNTP. 1 μl (equivalent to 20pM) of the LC-5 primer and 1μl (20 pM) of the 3' backward LC-2 primer was admixed into

15   the DNA solution. To the admixture, 73 μl of sterile water and 2.5 units of Taq DNA polymerase was added. Two drops of mineral oil were placed on top of the admixture and 35 rounds of PCR amplification in a thermocycler were performed. The amplification cycle

20   consisted of 52°C for 1 minute, 72°C for 2 minutes and 94°C for 0.5 minutes.

The resultant PCR modified b12 light chain DNA fragment was purified by electrophoresis in a 2.5% Nu-Sieve agarose gel (FMC). The area in the agarose

25   containing the modified b12 light chain DNA fragment was isolated from the agarose.

2)   Insertion of the Modified b12 Light Chain into the pEE12 Mammalian

30   Expression Vector

The modified b12 light chain purified PCR product and the pEE12 vector were digested with HindIII and EcoR I in separate reactions. The digested DNAs were analyzed on an LMPA gel, the DNA

35   excised, and ligated together in the presence of the

LMPA gel as described for the heavy chain construct in
Example 4d. The ligation products were transformed
into DH5α competent cells, minipreps analyzed, and DNA
prepared as described for the heavy chain constructs

5   in Example 4d.

f.   Insert<u>ion of the Modified b12 Heavy Chain</u>
<u>into the pEE12 Mammalian Expression Vector</u>
<u>Containing the b12 Light Chain to Create</u>
10   <u>the Combinatorial Vector pEe12 Combo BM 12</u>
A heavy chain cassette comprising the HCMV
promoter, enhancer elements, heavy chain gene, and
polyadenylation signal were removed from the pEE6
vector and inserted into the pEE12 vector containing
15   the b12 light chain gene, prepared in Example 4e, to
generate the combinatorial construct, pEe12 Combo BM
12, containing both the b12 light and heavy chain
genes (Figure 28).

The heavy chain cassette was removed from the
20   pEE6 vector by digestion with BglII and Sal I. The
pEE12 vector containing the light chain gene, prepared
in Example 4e, was also digested with BglII and Sal I.
The heavy chain cassette and the pEE12 vector
containing the light chain gene from Example 4e were
25   ligated together at the BglII and Sal I sites as
described in Example 4d. The combinatorial construct
was transformed into DH5α competent cells and miniprep
DNA was analyzed for the presence of the heavy and
light chains as in Example 4d. The nucleotide
30   sequence of the heavy and light chain genes was
determined. The nucleotide sequence of pEe12 Combo BM
12, the pEE12 vector containing the b12 heavy and
light chain genes is given in the sequence listing as
SEQ ID NO 156 and is illustrated in Figures 29A
35   through 29R.

g.   gp120 Binding of b12 IgG1 Antibody
      Expressed from the Heavy and Light Chain
      Genes in the Combinatorial Vector pEe12
      Combo BM 12

The combinatorial pEe12 Combo BM 12 vector
containing both the heavy and light chain genes was
used to transfect CHO cells. Stable clones were
selected in Glasgow Minimal Essential Media (GIBCO)
supplemented with 10% dialyzed fetal bovine serum and
50 μM methyl sulfoxamine (MSX). Several clones were
isolated and expanded in 6-well cluster dishes. The
supernatants of subconfluent cultures were harvested
and tested by ELISA for binding to gp120 as described
in Example 4c2. The clone producing the highest
levels of b12 IgG1 as determined by ELISA with gp120
IIIB was chosen for further study. The antibody was
purified by affinity chromatography using protein A as
described in Sambrook, et al., supra. The affinity of
b12 IgG1 for gp120 IIIB as measured by surface plasmon
resonance as described in Example 2b6c is
1.3×10^9 M^{-1}.

5.   Neutralizing Activity of Recombinant b12 Whole
      IgG1 Antibody (b12 IgG1) Against HIV-1 In Vitro

The key issue in producing antibodies to HIV-1
for therapeutic or prophylactic purposes is that they
should be highly potent (of high affinity and
neutralizing ability) and be cross reactive with a
wide range of primary clinical (field) isolates.
These are generally two opposing characteristics. The
ability of b12 whole IgG1 antibody (b12 IgG1) to
neutralize the infectivity of laboratory strains of
HIV-1 and a wide variety of primary clinical isolates
has been examined in p24 ELISA assays, microplaque
assays, and by syncytial formation assays.

The primary clinical isolates used as a source of
HIV-1 virus in these assays came from various regions
of the world by three organizations: the World Health
Organization (WHO), the Henry M. Jackson Foundation
for the Advancement of Military Medicine (HMJFAMM),
and the National Institute of Allergy and Infectious
Diseases (NIAID). Isolates from the WHO Network for
HIV-1 Isolation and Characterization were obtained
through the AIDS Research and Reference reagent
Program, Division of AIDS, NIAID, NIH. Isolates from
HMJFAMM were provided by Dr. John Mascola, Walter Reed
Army Institute of Research, Rockville, MD and Dr.
Francine McCutchan, Henry M. Jackson Research
Laboratory, Rockville MD. Isolates from NIAID were
kindly provided by Dr. Jim Bradac, Division of AIDS,
NIAID, NIH.

The HIV-1 viruses were collected from various
regions of the world, expanded in mitogen-stimulated
peripheral blood mononuclear cells (PBMC) (Mascola et
al., J. Infect. Dis., 169:48-54 (1994)), and culture
supernatants containing infectious virus were stored
in central repositories at -70°C. The designation of
viruses into clades was made on the basis of sequence
information based on the gag gene or on the V2-C5
region of gp120, or in some cases, after heteroduplex
mobility analysis (Louwagie et al., AIDS, 7:769-772
(1993) and Delwart et al., Science, 262:1257-1261
(1993)).

The HIV-1 viruses include a set of 14 primary
isolates which contain a high proportion of isolates
which are relatively refractory to antibody
neutralization by sera from other HIV-1 infected
individuals (Wrin et al., J. Acq. Imm. Def. Synd.,
7:211-219 (1994)), 12 primary infant isolates obtained
at birth or within two weeks of age, and 69

international isolates belonging to 6 different
clades.

Several different neutralization assays were
performed because HIV-1 neutralization by antibody
5   shows considerable variation depending upon the assay
used and the precise experimental conditions such as
inoculum size and incubation time of virus and
antibody (D'Souza et al., AIDS, 8:169-173 (1994)). By
performing neutralization assays on a range of
10   laboratory and primary isolates in a number of
different laboratories, it has been demonstrated that
b12 IgG1 is a highly potent neutralizing antibody
effective against a wide breadth of isolates.

15   a.   Quantitative Neutralization of HIV-1 MN and
            IIIb by b12 IgG1 as Measured in a Plaque
            Assay
            b12 IgG1 was initially tested for its
ability to neutralize the HIV-1 laboratory strains MN
20   and IIIB in a plaque formation assay in laboratories
which recently tested a panel of monoclonal antibodies
as part of the NIAID/WHO Antibody Serological Project
(D'Souza et al., supra).
            b12 IgG1 showed 50% neutralization titers of 3
25   ng/ml for the MN strain and 7 ng/ml for the IIIB
strain using plaque formation (Hanson, et al., J.
Clin. Microbiol., 28:2030-2034 (1990)) to determine
the ability of the antibody to inhibit infectivity of
the HIV-1 strains.
30

            b.   Quantitative Neutralization of HIV-1 MN and
                  IIIb by b12 IgG1 as Measured by Syncytial
                  Formation
                  b12 IgG1 showed 50% neutralization titers
35   of 20 ng/ml for both MN and IIIB strains using

syncytial formation as the reporter assay as described in Example 3b (Nara et al., <u>AIDS Res. Human Retroviruses</u>, 3:283-302 (1987)).

      The syncytial formation assay was performed as described in Example 5c. Briefly, virus was grown in H9 cells. For infectivity measurement, monolayers of CEM-SS target cells were cultured with 100-200 syncytial forming units (SFUs) of virus, in the presence or absence of antibody, and the number of syncytia determined after 3-5 days of incubation. The assays were repeatable over a virus-surviving fraction range of 1 to 0.001 within a 2 to 4-fold difference in the concentration of antibody (P<0.001).

    c.   <u>Neutralization of Primary Virus Isolates by</u>
        <u>b12 IgG1 as Measured by the p24 ELISA Assay</u>

      The ability of b12 IgG1 to neutralize infectivity of PBMCs by HIV-1 virus was quantitatively measured in the p24 ELISA assay (Daar et al., <u>Proc. Natl. Acad. Sci. U.S.A.</u>, 87:6574-6578 (1990) and Ho et al., <u>J. Virol.</u>, 65:489-493 (1991)). The p24 ELISA assay is further described in Example 3a.

    1)   <u>Neutralization of Ten Primary Virus</u>
        <u>Isolates by b12 IgG1</u>

      HIV-1 viruses were isolated from 10 individuals from various locations in the U.S. and with varying disease status. The HIV-1 viruses had been cultured only once or twice in peripheral blood mononuclear cells (PBMCs). Viral stocks were grown in PBMCs and the assay was performed in PBMCs.

      Briefly, HIV-1 virus at 50 $TCID_{50}$ and varying concentrations of b12 IgG1 were incubated together for 30 min at 37°C before addition to PHA-stimulated PBMCs. HIV-1 virus replication was assessed after

incubation for 5 to 7 days by p24 ELISA measurement as described in Example 3a. HIV-1 virus positive controls used in this assay were the molecularly cloned HIV-1 virus JR-CSF and the HIV-1 isolate JR-FL (O'Brien et al., J. Virol., 66:3125-3130 (1992), O'Brien et al., Nature, 348:69-73 (1990), and O'Brien et al., J. Virol., in press (1994)). Stocks of JR-CSF were prepared by infection of PBMC with supernatants initially obtained by DNA transfection. HIV-1 IIIB and HIV-1 MN are viruses with an extensive history of passage in transformed T-cell lines (Robert-Guroff et al., Nature, 316:72-74 (1985)). Stocks of these strains grown in H9 cells were passaged in mitogen-stimulated PBMC to prepare viruses that had been grown in the same cells as the primary viruses, to eliminate the influence of any host cell-dependent epigenic factors on virus neutralization (Wrin, et al., J. Acq. Imm. Def. Synd., 7:211-219 (1994)). The stock of PBMC-grown MN was a gift from A. N. Conley (Merck Research Labs).

2)   Neutralization of 12 Primary
          Infant Isolates by b12 IgG1

b12 IgG1 was also tested for the ability to neutralize infectivity of a panel of 12 primary infant isolates in the p24 ELISA assay. Virus isolates were obtained from 12 infants born to HIV-1 seropositive mothers; 7 were obtained at birth and 5 between birth and 14 days of age. All the infants were from California. Virus was isolated from patient PBMCs by coculture with PBMCs from healthy seronegative donors. Viral stocks were prepared by passaging the last positive culture dilution once into PBMCs. All of the isolates, except one (isolate 7), were non-syncytial inducing in MT2 cells and therefore

could not be assayed in the syncytial forming assay as herein described. HIV-1 virus from these stocks was grown in PBMCs and neutralization assessed using PHA-stimulated PBMCs as indicator cells and determination of extracellular p24 as the reporter assay essentially as described in Example 3a (AIDS Clinical Trials Group Virology manual for HIV Laboratories, Department of AIDS Research, NIAID, NIH, version 2.0 (1993)).

Serial dilutions of b12 IgG1 (0.3 to 20 μg/ml) were incubated with 20 $TCID_{50}$ or 100 $TCID_{50}$ virus in triplicate for 2 hours at 37°C before addition to PHA-stimulated PBMCs. Virus replication was assessed after 5 days by p24 ELISA measurement. Neutralization was expressed as either a 50% or 90% reduction in p24 antigen as compared to values observed in the absence of antibody (Table 6).

d.   Neutralization of Primary Virus Isolates by b12 IgG1 as Measured in a Microplaque Assay

A quantitative microplaque assay to measure the reduction of infectivity of primary clinical isolates of HIV-1 in the presence of the b12 IgG1 and pooled human plasma was performed as described in Hanson et al., J. of Clin. Microb., 2030-2034 (1990). The set of primary clinical isolates was chosen to contain a high proportion of isolates which are relatively refractory to antibody neutralization by sera from other HIV-1 infected individuals (Wrin et al., J. Acq. Imm. Def. Synd., 7:211-219 (1994)). Viruses were grown in PBMCs and the assay carried out in MT2 cells. This limits study to viruses which grow in this cell line but provides an additional measure of neutralization.

Primary clinical isolates of HIV-1 were isolated from frozen peripheral blood lymphocytes obtained from

seropositive donors as described in Gallo et al., _J._
of Clin. Microb., 1291-1294 (1987) and cultivated in
peripheral blood mononuclear cells (PBMC). Briefly,
HIV isolates were obtained by incubating frozen HIV-
infected patient PBMCs with seronegative donor PBMCs
in RPMI-1640 medium containing 20% heat-inactivated
fetal bovine serum, 2 μg/ml polybrene, 5% interleukin-
2, and 0.1% anti-human leukocyte interferon. The
cultures were fed with fresh donor PBMCs once a week,
and the supernatants were assayed for the presence of
reverse transcriptase (RT) activity beginning at day
11. The cultures were considered positive if, for 2
consecutive weeks, the RT counts were >10-fold higher
than those in the cultures of the seronegative donor
PBMCs alone.

The resultant RT-positive virus isolates were
tested for cytolysis in the MT4 (α-4 clone) (Hanson et
al., _supra_). Cytolysis in MT4 is a requirement for
viruses to be usable in the subsequent MT2 microplaque
assay system. Supernatant fluids from the primary
PBMC isolation cultures were used to infect expanded
cultures of phytohemagglutinin (PHA)-stimulated PBMCs
from healthy seronegative blood donors. These
infected PBMC cultures were grown in RPMI-1640 medium
supplemented with 15% fetal bovine serum, 5%
interleukin-2, 0.1% anti-α interferon, 2 μg/ml
polybrene, 50 μg/ml gentamicin, 100 U/ml penicillin,
and 100 μg/ml streptomycin. The crude supernatants
were harvested after 7 days and frozen as viral stocks
at -70°C.

The primary clinical isolates of HIV-1 used in
this microplaque assay are given in Table 6. VL134,
VL648, and VL025 are viruses isolated from infected
mothers in New York in 1992; UG266 and UG274 are
clade D isolates which were a gift from John Mascola

the Division of Retrovirology, Walter Reed Army
Institute of Research; the remaining viruses were
isolated from homosexual males in California in 1992.
The pooled human plasma preparation, containing
neutralizing antibody, was derived from 13 HIV-1
positive individuals selected for high neutralization
titer against the MN isolate. The laboratory HIV-1
strains MN and IIIb were propagated in H9 cells as
controls in the microplaque assay.

b12 IgG1 and a pool of human plasma from 13 HIV-1
seropositive patients were used as the source of
neutralizing antibodies in a 96-well microtiter plaque
reduction assay as described by Hanson et al., supra.
Briefly, 3-fold serial dilutions of the b12 IgG1 or
heat-inactivated pooled patients' plasma were combined
in quadruplicate with an equal volume containing 20
plaque-forming units (PFU) of HIV-1 virus per well and
incubated for 18 hours at 37°C. Negative control
wells also contained 50% normal human serum pool with
no patient immune serum. After the 18 hour incubation
of Fabs or serum and virus, 90,000 MT2 cells were
added per well and incubated at 37°C for 1 hour.
SeaPlaque Agarose in assay medium at 39.5°C was then
added to a final concentration of 0.8%. While the
warm agarose was still molten, the microtiter plates
were centrifuged at 20°C for 20 minutes at 500 X g to
form cell monolayers. The plates were incubated for 6
days at 37°C and then stained 18 to 24 hours with 50
μg/ml propidium iodide. The fluorescent plaques were
counted with transillumination by a 304 nm ultraviolet
light source using a low-power stereo zoom microscope.
Inhibition of infectivity, or neutralization titer, is
defined as the μg/ml of Fab or the plasma dilution
giving 50% inhibition of plaque count as compared with
controls without antibody. This dilution was

interpolated between data points.

     e.   <u>Results of the Neutralization Assays by b12</u>
            <u>IgG1 with Laboratory Virus Isolates</u>

Results of the ability of the b12 IgG1 to
neutralize laboratory virus isolates in both the
plaque and syncytial formation assays suggest the
antibody is approximately two orders of magnitude more
potent than other CD4 site antibodies in the WHO/NIAID
Project and comparable to the best antibodies directed
to the V3 loop of gp120. However, whereas antibodies
directed to the V3 loop of gp120 are strongly strain
specific, b12 IgG1 is roughly equally effective
against MN and IIIB. The b12 IgG1 antibody is
comparable in potency to a CD4-IgG molecule in these
assays (Example 3c). In a separate assay using p24
production to determine infectivity (Daar et al.,
<u>Proc. Natl. Acad. Sci. U.S.A.</u>, 87:6574-6580 (1990) and
Ho et al., <u>J. Virol.</u>, 65:489-493 (1991)), 50%
neutralization titers of less than 40 ng/ml were found
for both the MN and IIIB laboratory strains.

     f.   <u>Results of the Neutralization Assays by b12</u>
            <u>IgG1 with Primary Virus Isolates</u>

b12 IgG1 showed essentially complete
neutralization of 7 of 10 isolates at 5 μg/ml with all
the isolates showing 50% neutralization at ≤1 μg/ml as
determined in the p24 reporter assay (Figure 21).

The inhibition of infectivity, or neutralization
titer, for b12 IgG1 and the pooled HIV seropositive
human plasma from 13 donors is given in Table 6. The
neutralization titer for each of the viral isolates is
expressed as the minimum μg/ml of b12 IgG1 required
for 50% inhibition of plaque count as compared to the
controls. The neutralization titer for each of the

viral isolates is expressed as the minimum titer of
the pooled HIV seropositive human plasma from 13
donors required for 50% inhibition of plaque count as
compared to the controls.

5

Table 6

virus	host cell	b12 IgG1 50% neutralization titer (μg/ml)	pooled human plasma: dilution for 50% neutralization
IIIB	H9	0.007	1:767
MN	H9	0.003	1:24,000
VL135	PBMC	10	1:44
UG274	PBMC	0.7	1:37
VL134	PBMC	5.6	1:30
VL596	PBMC	8.5	1:17
UG266	PBMC	3.8	1:12
VL434	PBMC	22	1:10
VL172	PBMC	>200	1:10
VL750	PBMC	>200	1:10
VL069	PBMC	>50	<1:10
VL077	PBMC	>200	<1:10
VL114	PBMC	<7.4	<1:10
VL263	PBMC	5.0	<1:10
VL648	PBMC	16.7	<1:10
VL025	PBMC	16.7	<1:10

The b12 IgG1 was able to neutralize ten of the fourteen primary clinical isolates assayed at concentrations of ≤50 μg/ml as measured as the μg/ml required for 50% inhibition of plaque count as compared to the controls (Table 6). Pooled human plasma was able to neutralize 5 of the 14 primary clinical isolates assayed at >1:10 dilution as measured as the dilution required for 50% inhibition of plaque count as compared to the controls without antibody.

Table 6 shows that four isolates, which were not neutralized even by a 1:10 dilution of pooled human plasma, were neutralized by b12 IgG1. Most of the viruses reported in Table 6 were isolated from U. S. donors although two, both of which are neutralized by b12 IgG1, were from Ugandan donors and assigned to clade D.

Results of neutralization of 12 infant primary isolates with b12 IgG1 as determined by p24 ELISA measurements are given in Table 7.

Table 7

b12 IgG1
Antibody Concentration (μg/ml)

Infant Isolate	50% inhibition	>90% inhibition
1	20	>20
2	1.25	>20
3	<0.3	0.3
4	<0.3	0.6
5	2.5	20
6	5	>20
7	5	>20

8	<0.3	0.3
9	0.3	5
10	0.3	2.5
11	<0.3	0.6
12	<0.3	0.3

As shown in Table 7, b12 IgG1 achieved 90%
neutralization for 8 of 12 infant isolates at
concentrations of ≤20 µg/ml in the p24-based assay.
All 12 isolates were 50% neutralized in the range of
0.3 to 20 µg/ml with the majority being neutralized at
<5 µg/ml. In contrast, a pooled hyperimmune globulin
product HIVIG achieved 90% neutralization of only 3 or
12 isolates within a concentration range up to 100
µg/ml. HIVIG is a hyperimmune IgG preparation
obtained from the pooled plasma of selected HIV-1
asymptomatic seropositive donors meeting the following
criteria: presence of p24 serum antibody titers >128,
CD4 lymphocyte count ≥400 cells/µl and the absence of
p24 and hepatitis B surface antigen by enzyme
immunoassay (Cummins et al., Blood, 77:1111-1114
(1991)). The HIVIG used in these experiments was lot
number IHV-50-101 (North American Biologicals).

HIV-1 neutralization by antibody shows
considerable variation depending upon the assay used
and precise experimental conditions such as inoculum
size and incubation time of virus and antibody
(D'Souza et al., supra). However, by carrying out
neutralization on a range of laboratory and primary
isolates in a number of assays in different
laboratories, we have shown that b12 IgG1 is a highly
potent neutralizing antibody effective against a wide
breadth of primary isolates. The results clearly

demonstrate that, although primary isolates may be
more difficult to neutralize by antibody than
laboratory strains, they are not intrinsically
resistant (Conley et al., <u>Proc. Natl. Acad. Sci.</u>
<u>U.S.A.</u>, 91:3348-3353 (1994)). The potency of b12 IgG1
against the majority of U. S. isolates is in a
concentration range (≤ 5 μg/ml) which could be achieved
in vivo in passive immunotherapy. Furthermore, the
affinities of recombinant antibodies displayed on
phage can be enhanced by mutagenesis and selection in
vitro and this strategy has been used to considerably
improve the potency and breadth of reactivity of Fab
b12 (Barbas et al., <u>Proc. Natl. Acad. Sci., U.S.A.</u>,
91:3809-3812 (1994)). For optimal potency and strain
cross-reactivity for passive immunization, a cocktail
of in vitro improved antibodies may be most
appropriate.

The results have implications for passive
immunization and vaccine design. The ability of b12
IgG1 to neutralize a range of primary isolates implies
conservation of a structural feature associated with
the CD4 binding site of gp120 which is accessible to
antibody and important for neutralization. A vaccine
might seek to present this feature to the immune
system. Clearly, the feature is present on
recombinant gp120 since b12 was affinity selected from
a library using this molecule. However, b12 and
related antibodies formed only a small part of the
repertoire affinity selected from this library by
recombinant gp120. Most of the antibodies obtained
were far less potent in neutralization even though
they were also directed to the CD4 binding site, were
cross-competitive with b12 for binding to recombinant
gp120 and had similar affinities to b12 (Barbas et
al., <u>Proc. Natl. Acad. Sci., U.S.A.</u>, 89:9339-9343

(1992), Barbas et al., J. Mol. Biol., 230:812-823
(1993), and Example 2b6). Therefore, recombinant
gp120 appears to present the b12 epitope in
conjunction with several other weakly neutralizing and
overlapping epitopes and its efficacy as a vaccine may
suffer. Interestingly, evidence from antibody binding
to infected cells suggests that b12 does recognize a
native conformation of gp120 more effectively than
other CD4 binding site antibodies (Example 7). In any
case, b12 IgG1 and the library approach could be
useful in vaccine and passive immunization evaluation.
The ability of a candidate vaccine to preferentially
bind b12 and/or preferentially select potent
neutralizing antibodies from libraries should be
positive indicators for vaccine development.

5.   Determination of the Relationship Between the
      Epitopes Recognized by Fabs with Purified HIV-1
      Antigens

      The Fabs show a spectrum of neutralizing
abilities as described in Example 5. It was therefore
sought to determine if the epitopes recognized by
individual Fabs could be distinguished from each
other, and if possible, determine how the epitopes
recognized by the individual Fabs related to
neutralization.

      a.   Competitive ELISA between Fabs and b13
            Whole IgG1 Antibody for Binding to gp120

            The first method to distinguish between the
epitopes bound by the Fabs of this invention was to
compare the epitope recognized by the Fab b13 with the
other Fabs. The Fab b13 had been spliced to the Fc
region of IgG1 to generate a whole IgG1 molecule and
therefore contains the Fc region of the IgG1 antibody.

The other Fabs do not contain the Fc region of the
IgG1 antibody. The binding of the b13 IgG1 could
therefore be distinguished from the binding of other
Fabs by using a labeled anti-Fc reagent in competition

5   ELISA. A competition ELISA in which the Fabs b3, b6,
b11, b12, and b14 competed with b13 IgG1 for binding
to immobilized gp120 was performed.

Competitive ELISAs were performed between the
Fabs b3, b6, b11, b12, and b14 and the b13 whole IgG1

10   antibody. The whole antibody was obtained by splicing
constant domain genes with the b13 Fab and expressing
the protein in Chinese Hamster Ovary cells (CHO) as
described in Example 4 (Bender et al., _supra_ and in
Example 4a__ for the Fab b12). The ELISA was performed

15   as described above in Example 2b6b. Briefly,
microtiter wells were coated with 0.1 μg/ml of gp120
derived from the HIV-1 strain LAI in 0.1 M bicarbonate
buffer at pH 8.6. Soluble or free Fab fragments were
serially diluted from 1:100 to 1:32,000 in 0.5%

20   BSA/0.025% Tween 20/PBS. The dilution of b13 IgG1 was
held constant at 1:10,000 in 0.5% BSA/0.025% Tween
20/PBS. The b13 IgG1 and Fabs were admixed, added to
the gp120-coated microtiter wells and maintained for
120 minutes at 37°C. After maintenance, the wells

25   were carefully washed ten times with 0.05% Tween
20/PBS. The amount of b13 IgG1 antibody bound to the
plate after washing was detected using a peroxidase-
labeled antibody specific for the Fc portion of IgG1
contained on the b13 antibody.

30   Results of this assay indicated that the Fabs b3,
b6, b11, b12, and b14 are competitive with b13 IgG1
for binding to gp120 indicating that the epitopes
recognized by the individual Fabs are probably either
proximal or identical to the epitope recognized by the

35   b13 IgG1. A control anti-tetanus toxoid Fab did not

compete with IgG1 b13 in this assay.

Competition monitored in an ELISA format showed
that all of the Fabs compete with the b13 Fab as a
whole IgG. There is also an indication that Fabs b12

5   and b13 are distinct in that they are somewhat less
effective in cross-competition than the other members
of the panel.

     b.   Epitope Similarity Determination
            Between the Fabs in Binding to gp120

10   Using BIAcore

A more precise method for determining
the similarity of epitopes was performed using the
BIAcore. The procedure adopted here was to immobilize
a polyclonal anti-human $F(ab')_2$ on the sensor chip and

15   use this to capture the individual Fabs. An Fab of
this invention was injected and captured by the
polyclonal anti-human $F(ab')_2$. The captured Fab was
then used to bind gp120 derived from the HIV-1 strain
LAI. The captured Fab would thus bind the gp120 at

20   its respective epitope. A second Fab of this
invention was then injected. A response in the
BIAcore assay after injection of the second Fab
indicates that binding has occurred. If the second
Fab injected recognizes the same or similar epitope on

25   the gp120 as the first Fab, no response would occur.
No response would therefore indicate that the two Fabs
tested in the assay competed for binding to the same
or similar epitope on gp120. Alternatively, a
response in the assay suggests that the epitopes

30   recognized by the two Fabs are distinct from one
another and that binding of the second Fab to gp120 to
a second epitope is possible in the presence of the
first Fab. A response would therefore indicate that
the two Fabs tested in the assay did not compete for

35   binding to the same or similar epitope.

The precise epitope similarity determination with
the BIAcore was performed as follows. A flow rate of
5 μl/min of PBS, pH 7.4 was established and the
biosensor chip was activated by injecting 30 μl of

5   activation solution (Pharmacia Biosensor, 50% 0.2 M N-
ethyl-N'-(e-diethylaminopropyl)-carbodiimide, 50% N-
hydroxysuccinimide). The flow rate was then adjusted
to 10 μl/min and the antigen was injected in 10 mM
sodium acetate buffer, pH 4.5. Forty μl of goat anti-

10   human F(ab')$_2$ (Pierce) at a concentration of 40 μg/ml
in 10 mM sodium acetate buffer, pH 4.5 was injected to
give a final immobilization of 10000 Response Units
(RU). The chip was then blocked from any further
immobilization by injecting 30 μl of 1 M ethanolamine,

15   pH 8.5 (Pharmacia Biosensor). The flow rate was
adjusted to 1 μl/min and 4 μl of the first Fab at a
concentration of 100 μg/ml was injected, immediately
followed by 4 μl of an anti-cytomegalovirus Fab at a
concentration of 150 μg/ml to block any remaining

20   binding sites on the immobilized goat anti-human
F(ab')$_2$. Next, 4 μl of gp120 at a concentration of 10
μg/ml was injected followed by 4 μl of the second Fab
at 100 μg/ml. The assay was performed with a
combination of all of the Fabs to give a mosaic of

25   binding patterns. The entire surface was regenerated
with 25 μl of 60 mM HCl so that the next cycle could
be performed.

Table 8b indicates the results of the epitope
similarity determination by BIAcore. Table 8a shows

30   the positive and negative controls for the clones
used. The positive controls are the RU levels
obtained when the first Fab used is the clone
indicated and the second Fab is an anti-gp120 V3-loop
Fab. The Fabs of this invention compete with soluble

35   CD4 for binding to gp120. The second Fab, an anti-

gp120 V3-loop Fab, neither competes with soluble CD4
nor competes with anti-CD4 site Fabs and therefore
would react with a different epitope than the Fabs of
this invention. As can be seen from the table, all
5   positive controls result in significant values of 125
or more, indicating the validity of the technique to
distinguish between non-identical epitopes. The
negative controls are the values obtained when the
same Fab is injected twice. This gives the background
10   values for each Fab. These values were subtracted
from all subsequent experiments in order to give true
values.

An epitope map, Table 8b, was then constructed.
ND indicates that this combination of Fabs was not
15   performed. It can be seen from this map that Fabs b3,
b6, b11, and b14 form a set which compete highly
effectively with one another for binding to a similar
or the same epitope. For the most part, a member of
the set competes for binding as well with another
20   member as it does with itself (RU = 0). On the other
hand, b12 and b13 appear somewhat different in that
while they compete for binding with members of the
above set, they do not compete as effectively as the
other Fabs within the set. Further, competition for
25   binding to the same or similar epitope between b12 and
b13 is incomplete. This suggests that the epitopes of
Fabs b12 and b13 are sufficiently dissimilar from
those of the other four and from each other, to allow
detectable binding when they are used in combination
30   with any of the other Fabs. It may therefore be
concluded that clones b3, b6, b11, and b14 bind the
same or similar epitopes, with Fabs b12 and b13 bind
to epitopes which can be distinguished from the other
epitopes in this assay.

35

Table 8a

Fab	b3	b6	b11	b12	b13	b14
POSITIVE CONTROL (RU)	129	128	131	125	135	134
NEGATIVE CONTROL (RU)	24	38	ND	17	15	ND

ND indicates that this combination of Fabs was not performed.

Table 8b

		Fab 1			
		b13	b12	b6	b3
	b14	30	24	14	0
Fab 2	b11	54	28	14	0
	b3	26	29	0	0
	b6	21	17	0	ND
	b12	22	0	ND	ND

ND indicates that this combination of Fabs was not performed.

c.   Comparison of Fab Epitopes with Wild-type
and Mutant Forms of gp120 Using ELISA with
gp120 in the Solid Phase

Epitope similarity determinations of the panel of Fabs was performed with a panel of HXBc2 gp120 mutants of the HIV-1 strain LAI. Conserved residues of gp120 were altered to generate the HXBc2 gp120 mutants. The interaction between the mutants

and Fabs was investigated to examine binding
specificity differences between the Fabs at greater
resolution.   The HXBc2 gp120 mutants used in this
assay had been previously characterized with respect

5   to gp160 precursor processing, gp120-gp41 association,
and CD4 binding ability (Olshevsky et al., <u>J. Virol.</u>,
64: 5701-5707 (1990)).   Both wild type and mutant
gp120s were tested for their ability to bind a
saturating concentration of each Fab.

10   The epitope determination with wild-type and
mutant gp120 was performed with HIV-1 envelope
glycoproteins from culture supernatants of COS-1 cells
transfected with plasmids expressing either wild-type
or mutant gp120 from the HXBc2 clone.   Microtiter

15   wells were coated with the antibody D7324 (Aalto
BioReagents; Dublin, Ireland) which binds to the
conserved 15 amino acid sequence at the carboxy
terminus of gp120.   The wild-type or mutant gp120 were
thus captured onto the surface of microtiter wells by

20   binding to the D7324 antibody.   A reference HIV-1
positive human serum pool at a 1:3000 dilution in 0.5%
Tween 20 was assayed for binding to the wild-type and
mutant gp120s by incubating the serum pool with the
immobilized gp120.   The bound antibody was detected by

25   a second enzyme conjugated antibody.   The reading
obtained with the HIV-1 positive human serum pool,
N=4, was used as the reference value for each mutant.
The Fabs of this invention were then assessed for
binding to the wild-type and mutant gp120s and the

30   ratio of the Fab to reference serum was determined for
each gp120 mutant (Table 9).   The average ratio for
the entire panel of Fabs was calculated and any
individual ratio deviating from the mean by less than
0.5 times was considered to indicate a gp120 amino

35   acid change that decreased Fab recognition, while

those deviating by more than 2.0 times indicated an
amino acid change that enhanced Fab recognition. In
this way, a map of mutations affecting the binding of
the Fab to gp120 was obtained for each clone

5   essentially as previously described (Helseth et al.,
J. Virol., 65:2119-2123 (1991) and Olshevsky et al.,
supra).

Table 9

Mutation	Fab					
	B3	B6	B11	B12	B13	B14
45 W/S	1.60	0.61	0.50	0.68	1.20	0.28
113 D/A	1.46	1.73	1.89	1.13	0.99	0.00
113 D/R	1.40	1.50	1.61	0.67	0.71	0.00
NO V1/V2	1.07	1.48	1.42	0.23	0.86	1.68
NO V1/V2/V3	2.05	1.48	1.94	0.47	0.95	1.60
NO V3	1.88	1.64	1.92	0.46	1.08	1.72
183/184 PI/SG	0.82	0.73	0.69	0.33	0.92	0.32
207 K/W	1.15	1.57	1.19	2.54	1.30	1.36
252 R/W	1.58	1.52	1.58	1.65	1.39	2.04
256 S/Y	0.64	0.14	0.33	0.82	1.15	0.00
257 T/R	0.08	0.59	0.00	0.76	0.22	0.00
257 T/A	0.86	0.93	0.75	0.99	0.68	0.40
257 T/G	0.91	0.70	1.14	0.74	0.75	0.00
262 N/T	1.06	0.64	1.19	0.62	0.72	0.24
269 E/L	0.73	0.48	0.45	0.78	0.83	0.20
314 G/W	0.59	0.36	0.39	0.65	0.71	0.28

Mutation	Fab					
356 N/I	0.67	0.66	0.39	0.92	0.80	0.52
368 D/R	0.19	0.18	0.00	0.04	0.00	0.00
368 D/T	0.28	0.20	0.00	0.03	0.02	0.00
370 E/R	0.01	0.25	0.17	0.07	0.00	0.00
370 E/Q	0.25	0.89	0.58	0.46	0.14	0.00
384 Y/E	1.21	1.02	1.11	0.25	0.02	0.88
386 N/Q	0.88	0.59	0.31	1.05	0.01	0.36
395 W/S	0.92	0.59	0.47	1.00	1.05	0.12
427 W/S	1.57	1.11	1.53	0.63	0.98	0.00
435 Y/S	1.93	1.16	1.58	1.41	1.24	2.04
450 T/N	0.62	0.48	0.58	0.75	0.75	0.60
457 D/A	0.62	0.39	0.44	0.28	0.62	0.20
457 D/R	0.84	0.55	0.92	0.32	0.58	0.56
470 P/L	0.80	0.64	0.72	0.72	0.18	0.24
475 M/S	0.06	1.02	0.33	1.50	1.39	0.92
477 D/V	0.50	0.09	0.00	0.07	0.52	0.00

The general patterns observed are broadly similar to many CD4 site antibodies and of soluble CD4. Fab b12 is distinguished by its decreased binding to a mutant in which the V1 and V2 loops are deleted. This may or may not be related to the enhanced neutralizing ability of Fab b12. However, it is clear that the V1 and V2 loops and the V3 loop can affect antibody binding to the CD4 binding site either by direct contact or by transmitted conformational effects.

Sensitivity to certain mutations in residues,

particularly towards the C-terminus of gp120, has previously been associated with CD4 binding site antibodies (Thali et al., J. Virol., 66:5636-5641 (1992) and Thali et al., J. Virol., 65:6188-6193 ((1991)).   These mutations include residue 257 mutated from threonine to arginine (257 T/R), 368 D/R, 370 E/R, 457 D/A and 477 D/V. Most of these mutations abrogate Fab binding or reduce it to low levels consistent with the assignment of the recombinant Fabs in this assay as reacting with the CD4 site.

In a particular mutant of gp120, the V1/V2 loop (residues 119-205) is completely removed. This mutation enhances the binding of Fabs b6, b11, and b14 but significantly decreases the binding of Fab b12. Deletion of the V3 loop produces a more modest decrease in Fab b12 binding while generally enhancing the binding of the other Fabs. The 314 G/W change in the V3 loop produces a decrease in binding of all the Fabs. This effect has been observed for other CD4 binding site antibodies (Moore and Sodroski, unpublished observations).

When the binding specificities of each Fab is examined in detail, each Fab has a unique mutant binding profile. For example, Fab b14 binding is eliminated by the 113 D/A change whereas the binding of the other Fabs is unchanged or enhanced; Fab b3 and b11 binding is reduced by the 475 M/S mutation but binding by the other Fabs is unchanged and the 370 E/Q change reduces binding of all the Fabs except for b6 and possibly b11. Fab b12 is distinguished by its decreased binding to a mutant in which the V1 and V2 loops are deleted. This may or may not be related to the enhanced neutralizing ability of Fab b12 and will be the subject of further study. However, it is clear that the V1 and V2 loops and the V3 loop can affect

antibody binding to the CD4 binding site either by direct contact or transmitted conformational effects.

The effects on Fab binding of a series of point mutations in gp120 afford the opportunity to look more closely at recognition differences. The general patterns observed are broadly reminiscent of many CD4 site antibodies and of soluble CD4 itself. Fab b12 is distinguished by its decreased binding to a mutant in which the V1 and V2 loops are deleted. This may or may not be related to the enhanced neutralizing ability of Fab b12. It will be necessary to study a number of variants of Fab b12, which could be produced by chain shuffling or mutation, to answer this question. However, it is clear that the V1 and V2 loops and the V3 loop can affect antibody binding to the CD4 binding site either by direct contact or transmitted conformational effects.

6.   Determination of the Relationship Between the
     Epitopes Recognized by the Fabs with HIV-1
     Antigen Multimeric Complexes
     a.   Comparison of Fab Epitopes with gp120 and
          gp160 Expressed as Multimeric Complexes on
          the Surface of COS-1 Cells

Given the lack of correlation of Fab neutralization with binding parameters assessed using recombinant gp120, the binding of Fabs b3, b6, and b12 to COS-1 cells expressing the HXBc2 envelope glycoproteins gp160 and gp120 was compared. Fab b3, the poorest neutralizer, Fab b6, also a poor neutralizer, and Fab b12, the most effective neutralizer as determined in Example 5 were used in the assay. The envelope glycoproteins expressed by the COS-1 cells were gp160, the precursor of gp120 and gp41, and the mature gp120. In this assay, different

concentrations of Fab were incubated with radiolabeled
COS-1 cells which express gp160 and gp120 on their
surface. The cells were then washed and lysed. The
gp120 and gp160 envelope glycoproteins bound to Fab

5   were precipitated with goat anti-F(ab')$_2$ antibody and
analyzed by protein gel electrophoresis and shown in
Figure 20. Since the amount of HIV-1 envelope
glycoprotein expressed on the surface of transfected
COS-1 cells is small compared with the amount present

10   intracellularly, after cell lysis, the bound Fab is
presented with a large excess of both mature gp120 and
gp160 precursor forms. The total amount of envelope
glycoproteins precipitated thus provides an indication
of the amount of Fab bound to the cell surface.

15   Scanning densitometry profiles were derived from the
autoradiographs and are expressed in arbitrary
densitometric units.

     Although the lack of saturation for Fabs b6 and
b3 precludes a precise estimate of affinity, it is

20   clear that Fab b3 exhibits a lower affinity for the
precursor gp160 than either Fab b6 or b12. When the
binding of Fab b12 and b6 are compared, several
differences are apparent. Assuming that Fab 6
achieves saturation at concentrations slightly higher

25   than 150 μg/ml, the estimated affinities of Fab b12
and b6 for the total population of envelope
glycoproteins recognized differ only marginally. The
most striking difference in the binding of Fab b12 and
b6 to the multimeric envelope glycoprotein complex is

30   the preferential detection of gp120 relative to gp160
by Fab b12. Using densitometry to estimate amounts,
it is seen from Figure 20 that Fab b12 immunoreacts
with an amount of gp120 that is at least about 50 %
more than the gp160 present in the immunoreaction

35   admixture. The estimated affinities, based on the Fab

concentrations at which half-maximal binding to gp120 is observed, are 3×10^7 M^{-1} and $<6 \times 10^6$ M^{-1} for Fabs b12 and b6, respectively.

The binding of the Fabs to the multimeric envelope glycoprotein complex on the transfected COS-1 cell surface provides some insights into the observed differences in neutralization potency. The binding of the most potent neutralizing Fab, Fab b12, achieves saturation at roughly 100 μg/ml, whereas neither of the less potent neutralizing Fabs achieves saturation even at 150 μg/ml. Fab b3 clearly exhibits a lower affinity for the cell surface envelope glycoprotein complex than do the other two Fabs tested, b12 and b6. The most striking difference in the binding of b12 and b6 to the multimeric envelope glycoprotein complex is the preferential precipitation of gp120 relative to gp160 by the bound Fab b12. In addition to these differences in gp120 recognition, it appears that the overall number of cell surface envelope glycoproteins capable of being recognized by the less neutralizing Fabs is greater than that seen for Fab b12. These differences suggest that Fab b12 may recognize a more limited subset of envelope glycoprotein conformations and that these conformations are better approximated by the mature gp120 glycoprotein in the cell lysates. It is known that the gp160 precursor assumes a greater variety of conformations during the maturation process than does the fully folded gp120 product (Thiriart, et al., J. Immunol., 143:1832-1836 (1989) and Fennie and Lasky, J. Virol., 63:639-646 (1989)). The enhanced neutralization ability of Fab b12 could reflect a higher affinity for a restricted gp120 conformation present in the functionally relevant subset of envelope glycoprotein spikes. Such a functionally relevant group of envelope glycoproteins moieties

probably represents a small subset of the total
population, consistent with the low infectious
fraction associated with HIV-1 and other retroviral
virus preparations. One caveat to these observations

5   is that the glycosylation of gp120 expressed as a
recombinant protein in baculovirus or on the surface
of COS-1 cells is likely to differ and this could
affect binding of the Fabs of this invention.
However, no difference in the affinity for CD4 binding

10   site antibodies between the two forms of gp120 has
been observed previously using a range of antibodies
(Moore and Sodroski, unpublished observations). In
addition, these studies employed a molecular clone of
HIV-1 and its extension to primary isolates will need

15   to be studied further.
     Fabs derived from combinatorial libraries may be
viewed as "artificial". However, as shown here, the
recognition properties of a set of antibodies directed
to the CD4 site of gp120 show many features in common

20   with those derived by conventional means. They also
show many features in common with one another
suggesting that, with the caveats inherent in the
library approach (Barbas et al., J. Molec. Biol.,
230:812-823 (1993) and Burton and Barbas, Nature,

25   359:782-783 (1992)), one individual produces several
clearly distinct antibodies directed to a common
structural feature, i.e., the CD4 binding site. This
is in agreement with observations made on anti-CD4
binding site antibodies using anti-idiotype antibodies

30   (Chamat et al., J. Immunol., 149:649-654 (1992) and
Hariharan et al., J. Virol., 67:953-960 (1993)). One
advantage of producing several antibodies is that
escape (at least in binding terms) is made more
difficult. The only mutations in Table 9 which

35   essentially eliminate the binding of all the

antibodies also reduce CD4 binding ability.

The observations presented here have significance for vaccine development. The most effective vaccine may need to induce antibodies to the CD4 binding site with properties similar to those of Fab b12. Given the data above, recombinant gp120 offers no special qualities in this regard. Further, the Fab b12 type of antibody formed only about 10% (4/33 Fabs) of the cloned response of the library donor (Barbas et al., J. Molec. Biol., 230:812-823 (1993)) and has not been described amongst the human antibodies derived by other means suggesting it may be a minor component of typical responses. It is clearly of some interest for vaccine design to define more precisely the structure recognized by Fab b12.

7.   Recognition of gp120 from Primary HIV-1 Isolates
      by b12 IgG1 in Vitro

The ability of the b12 IgG1 to recognize the gp120 molecule from HIV-1 virus from 69 primary isolates was determined in an ELISA assay. Recognition of the primary HIV-1 virus isolate with b12 IgG1 is indicative of the prevalence of the b12 epitope in the HIV-1 pandemic. To probe the occurrence of the b12 epitope in the HIV-1 pandemic, binding of the b12 IgG1 to gp120 from 69 international isolates belonging to 6 different clades was examined. Virus isolates assayed were obtained from the WHO, HMJFAMM, and NIAID.

Infectious culture supernatants containing virus and free gp120 were treated with 1%(v/v) Nonidet-P40 (NP40) non-ionic detergent to provide a source of gp120 (Moore et al., AIDS, 3:155-160 (1989)). Microplate wells (Immulon II, Dynatech, Ltd.) were first coated with sheep polyclonal antibody D7324.

This antibody was raised to the peptide
APTKAKRRVVQREKR, derived from the C-terminal 15 amino
acids of the clade B IIIB HIV-1 viral isolate. Next,
an appropriate volume of inactivated supernatant

5   containing gp120 was diluted with a buffer comprising
tris-buffered saline (TBS)/1% (v/v) NP40/10% fetal
calf serum (FCS) and a 100 μl aliquot added to the
microplate wells for 2 hours at room temperature.
Unbound gp120 was removed by washing with TBS, and

10 bound gp120 was detected with CD4-IgG (1 μg/ml) or
with b12 IgG1 diluted in a buffer comprising
TBS/2%(w/v) nonfat dry milk powder/20%(v/v)sheet serum
(TMTSS) essentially as previously described (Moore et
al., AIDS, 4:307-310 (1990)) and Moore et al., J.

15 Virol., 68:469-473 (1994)). CD4-IgG is a fusion
molecule which consists of CD4 and IgG. The CD4
portion binds to gp120 and the IgG portion provides
the means for detection of the CD4-IgG fusion molecule
with labeled anti-IgG reagents. Bound antibody was

20 then detected with an appropriate alkaline-phosphatase
conjugated anti-IgG, followed by AMPAK (Dako
Diagnostics). Absorbance was determined at 492 nm
(OD_{492}). Each virus was tested against CD4-IgG in
triplicate and against b12 IgG1 in duplicate. All

25 OD_{492} values were corrected for non-specific antibody
binding in the absence of added gp120 (buffer blank).
The mean, blank-corrected OD_{492} values for CD4-IgG and
b12 IgG1 were then calculated, and the OD_{492} ratios of
b12 IgG1:CD4-IgG were determined. This normalization

30 procedure enables allowance to be made for the
different amounts of gp120 captured onto the solid
phase via antibody D7324 when comparing antibody
reactivity with a panel of viruses. Binding ratios of
0.50 or greater were deemed to represent strong

35 antibody reactivity; ratios from 0.25-0.50 were

considered indicative of moderate reactivity;   values
of <0.25 were designated as representative of
essentially negative monoclonal antibody reactivity.

     As shown in Figure 22, b12 IgG1 reacts with ≥50%
of clades A-D but only 1 of 12 isolates from clade E.
Reactivity with clade B isolates from the U.S.A. is
approximately 75%.

8.   <u>Nucleic Acid Sequence Analysis Comparison Between
HIV-1 Specific Monoclonal Antibody Fabs and the
Corresponding Derived Amino Acid Residue Sequence</u>
     To explore the relationship between neutralizing
and weakly or non-neutralizing Fabs, the variable
domains of 32 clones expressing human anti-gp120 Fabs,
prepared in Example 2 including the 20 listed in
Figure 6 for which neutralizing activity was assessed,
were sequenced.   In addition, the five gp41-specific
Fabs were also sequenced.

     Nucleic acid sequencing was performed on
double-stranded DNA using Sequenase 1.0 (USB,
Cleveland, OH) and the appropriate primers hybridizing
to sequences in the Cg1 domain (SEQGb : 5′
GTCGTTGACCAGGCAGCCCAG 3′ SEQ ID NO 49) or the Ck
domain (SEQKb : 5′ ATAGAAGTTGTTCAGCAGGCA 3′ SEQ ID NO
50).   Alternatively sequencing employed single
stranded DNA and the T3 primer (5′ ATTAACCCTCACTAAAG
3′, SEQ ID NO 51) or one hybridizing to a sequence in
the Ck domain (KEF : 5′ GAATTCTAAACTAGCTAGTTCG 3′ SEQ
ID NO 52).

     The amino acid residue sequences of the variable
heavy and light chains derived from the nucleic acid
sequences of the 32 gp120-specific clones are shown
respectively in Figures 10 and 11.   Groupings are made
on the basis of similarities in heavy chain sequences.
Dots indicate identity with the first sequence in each

section.   The SEQ ID NOs are listed to the right of the corresponding derived heavy and light chain (V_H from SEQ ID NO 53-81 and V_L from SEQ ID NO 82-113) amino acid residue sequences in the Figures

5   themselves.

Alignment of derived sequences with one another and with the Genbank database made use of the MacVector suite of programs.   For analysis of heavy chain CDR3 sequences as described by Sanz, J.

10   Immunol., 147:1720-1729 (1991), the most 5' nucleotide was considered to be the first nucleotide after codon 95 of the H chain variable region according to Kabat et al, Sequences of Proteins of Immunological Interest, US Dept. of Health and Human Services,

15   Washington, DC (1991).   The most 3' nucleotide was assigned to the last unidentified nucleotide before the sequence matched with the published germline JH genes.   The CDR3 sequences were analyzed using the DNASTAR software.   Sequence comparisons were performed

20   with both the ALIGN and COMPARE programs in order to determine the germline D gene which provided the best homology throughout.   In a second step, the SEQCOMP program was used to find sequence identity of at least six nucleotides with either the coding strand or the

25   reverse complement of germline D genes.

The heavy and light chain sequences of the gp41-specific Fabs are shown in Figures 18 and 19, respectively.   The amino acid residue sequence of the CDR3 heavy chain exhibits the most variation between

30   the Fabs than any other region of the variable domain.

a.   <u>Organization of Antibodies into Groups</u>
      <u>According to Heavy Chain Sequence</u>

35   V_H and V_L domains of 32 gp120 clones were

sequenced and the V_H domains compared using MacVector software. This analysis immediately established that a number of the clones, including those selected by panning against different antigens, are closely

5   related to one another. The exception to this is the Fabs selected by panning against the V3 loop peptide which are not related to the Fabs selected by panning against the gp120/160 antigens. Figure 10 shows that the V_H sequences derived from gp120/160 panning can be

10   organized into 7 groups. The broad features apparent from a comparison of amino acid sequences are discussed herein.

The relatedness of sequences within a group varies considerably. For instance, in the group

15   beginning with clone number b8 the amino acid sequences are very similar. Six clones were identical and the remainder showed a maximum of 5 differences from the predominant sequence (the EQ difference due to the 5' primer excluded). Only one clone showed a

20   single difference in the CDR3 region. The average discrepancy over all the sequences in this group from the predominant sequence is 1.1 amino acid residues/ variable domain. This amount corresponds to the order of magnitude of discrepancies which could arise from

25   the PCR. Sequencing of constant domains indicated a PCR error frequency of about 1 base change per domain.

In contrast, in the group headed by clone b3, no two clones were absolutely identical. The average difference from the consensus group sequence is 3.3

30   residues per sequence and determination for the CDR3 alone is 1.3. Therefore, it seems likely that the heavy chains in this group are somatic variants of one another.

The group headed by clone 1 presents a third

35   pattern. Clones b1 and b14 are identical as are

clones b2 and B2. However, 23 amino acid differences
exist between the two sets of clones. Clones b24 and
B30 are approximately equally well differentiated
(13-25 differences) from either of these two sets of
clones or one another. Still the CDR3 regions are
very similar. A number of explanations can be
suggested for this pattern: 1) all clones in this
group originate from the same germline gene which has
undergone extensive somatic mutation, 2) cross-over
events have occurred to essentially recombine
different germline genes with the same DJ combination,
3) a "convergent evolution" process has led to the
selection of different germline genes associated with
the same DJ combination.

b.   Sequences of the V_L Domains from the gp120
      Binders

The V_L sequences of the Fabs were organized
into the groups defined in Figure 10 are shown in
Figure 11. Immediately apparent was the extensive
chain promiscuity as evidenced by the pairing of
different light chains with the same or a very similar
heavy chain with retention of antigen binding
capability and indeed, for the most part, antigen
affinity as compared with Figure 10. This promiscuity
can be explored further by reference to the groups
considered above.

The clone b8 group, in which the heavy chain
members were identical or very similar, also produced
4 light chains which are identical or very similar
(less than 3 amino acid differences). Therefore a
predominant heavy-light chain combination can be
described for this group. One member (clone b8) had
the same or very closely related V_L gene but appeared
to use a different Jk gene. Two other members (clones

B8 and b18) were more distantly related to the major
sequence (7-12 differences). Two further clones (b13
and B26) used a Vk gene from a different family, Vk3
compared to Vk1, and therefore were unrelated to the

5   major sequence.

The clone b3 group, suggested to contain somatic
variants of a single heavy chain, showed considerable
light chain diversity with no two members being
closely related to one another. Vk3-Jk2 combinations

10   predominated but Vk3-Jk3 and Vk1-Jk3 combinations also
occurred.

On the other hand, in the clone b1 group evidence
existed for the heavy chains being more choosy about
their light chain partner. Thus, closely related

15   heavy chains appeared to be paired with related light
chains. The identical heavy chain pairs (b1 and b14;
b2 and B2) had very similar light chains (2 and 4
amino acid differences respectively) whereas the
distinct heavy chains (b24 and B30) had distinct light

20   chains which were unrelated to one another or the
other group members. The clone 4 group provides
another example of this phenomenon in that 4 closely
related heavy chains were paired with 3 closely
related light chains (a predominant heavy-light chain

25   combination), except for the clone b7 light chain that
was distinct.

In summary, the heavy chain (V_H) sequences was
organized into 7 groups where each member of a group
has an identical or very similar CDR3 region with a

30   limited number of differences elsewhere. When the
light chains (V_L) were constrained into the groupings
defined by their heavy chain partners, considerable
light chain sequence variation was observed. This
phenomenon of chain promiscuity has been observed

35   previously and can be appreciated by reference to

Figure 11. Marked neutralizing ability was confined
to two groups of sequences. The first group consisted
of Fabs 4, 7, 12 and 21 which have very similar heavy
and light chains. The second group consisted of Fabs

5   13, 8, 18, 22 and 27. Only Fab 13 showed marked
neutralizing ability, although the others showed some
weaker activity. Interestingly in this group Fab 13
did have a light chain distinct from the other members
of the group.

10

9.   Shuffling of the Heavy and Light Chain of a
      Single Clone Against the Library

To further explore possible functional
heavy-light chain combinations, the heavy chain of

15   clone b12 (also referred to as Fab 12 for the
corresponding soluble Fab preparation) shown in Figure
10 was recombined with the original light chain
library prepared in Example 2 to construct a new
library H12-LCn. In addition, the b12 light chain was

20   recombined with the original heavy chain library to
construct a library Hn-L12. These two libraries were
taken through 3 rounds of panning against gp120 (IIIB)
as described in Example 2b5). The Fabs expressed from
the resultant immunoreactant clones were analyzed as

25   described in Example 3 above. Clone b12 was chosen as
this Fab neutralized HIV-1 in vitro as shown in
Example 3.

To accomplish the preparation of a shuffled
library from the Fd gene of clone b12 with the

30   original light chain library, the b12 heavy chain was
first subcloned into a tetanus toxoid binding clone
expressed in pComb2-3. The light chain library was
then cloned into this construction to give a library
of 1×10^7 members. The subcloning step was used to

35   avoid contamination with and over-representation of

the original light chain. A similar procedure was adopted for shuffling of heavy chains against the light chain from clone b12 to give a library of 3 x 10^6 members. Cloning and panning procedures were

5 carried out as described above for the original library.

Eleven light chains which recombined with the b12 heavy chain and bound gp120 by panning were randomly chosen for subsequent competition ELISA and sequence

10 analysis. The apparent affinities of these shuffled combinations were similar with an IC_{50} of approximately 10^{-8} to 10^{-9} M. The sequences were organized where a set of 3 were very similar to the original b12 light chain and the other 8 showing many

15 differences from the original with some sub-grouping possible.

The sequences of the light chains which bound to the b12 heavy chain clone are shown in Figure 12. The sequences are compared to the sequence for the

20 original light chain from clone b12. The light chains are identified by numbers which do not correspond to the original light chain clones; the assigned numbers of the newly selected clones having new light chains are thus arbitrary. The sequences of these light

25 chains are also listed in the Sequence Listing from SEQ ID NO 114 to 122. Some light chain sequences are identical. In addition to immunoreactivity with gp120, the new Fabs isolated from these shuffled clones were tested in the syncytia assay for

30 neutralization of HIV-1 infection as described in Example 3. Four shuffled monoclonal Fab antibodies, each having the heavy chain from clone b12, a known HIV-1 neutralizing clone, and new light chains designated L28, L25, L26 and L22, all exhibited

35 approximately 60% neutralization in a syncytia assay

with 0.4 μg/ml purified Fab. This effect was
equivalent to that obtained with the original clone
b12 heavy and light chain pair. Maximum
neutralization of approximately 80% was obtained with

5   the H12/L28 and H12/L25 Fabs at 0.7 μg/ml which was
equivalent to that seen with the original clone b12
heavy and light pair. The neutralization resulting
from the H12/L22 and H12/L26 Fabs plateaued at 60%
with Fab concentrations of 0.4 μg/ml up to 1.0 μg/ml.

10   Thus, in addition to the gp120 immunoreactive and HIV
neutralizing Fabs obtained in the original library
prepared as described in Example 2, by shuffling a
known neutralizing heavy chain with a library of light
chains, new HIV-1 neutralizing Fab monoclonal

15   antibodies have been obtained.

     Ten heavy chains which recombined with the b12
light chain were also randomly chosen. One was very
similar to the original b12 heavy chain but the others
have many differences. Nevertheless, the V-D and D-J

20   junctions were essentially identical indicating the
clones had probably arisen from the same rearranged
B-cell clone by somatic modification. Competition
ELISA failed to reveal any clear difference in
affinity between the variants selected from those

25   originally analyzed.

     The sequences of the heavy chains which bound to
the b12 light chain clone are shown in Figure 13. The
sequences are compared to the sequence for the
original heavy chain from clone b12. The heavy chains

30   are identified by numbers which do not correspond to
the original light chain clones; the assigned numbers
of the newly selected clones having new heavy chains
are thus arbitrary. The sequences of these light
chains are also listed in the Sequence Listing from

35   SEQ ID NO 123 to 132. Some light chain sequences are

identical. In addition to immunoreactivity with
gp120, the new clones were tested in the syncytia
assay for neutralization of HIV-1 infection as
described in Example 3. Two shuffled monoclonal Fab

5   antibodies, each having the light chain from clone
b12, a known HIV-1 neutralizing clone, and new heavy
chains designated H2 and H14, exhibited approximately
40% neutralization in a syncytia assay with 1.0 and
0.5 μg/ml purified Fab, respectively. This effect was

10   equivalent to that obtained with the original clone
b12 heavy and light chain pair at a concentration of 2
μg/ml. Maximum neutralization of approximately 50%
was obtained with the Fab having the new H14 chain at
1.0 μg/ml compared to 80% neutralization with 0.7

15   μg/ml with the original clone b12 heavy and light
pair. Thus, in addition to the gp120 immunoreactive
and HIV neutralizing Fabs obtained in the original
library prepared as described in Example 2, by
shuffling a known neutralizing light chain with a

20   library of heavy chains, new HIV-1 neutralizing Fab
monoclonal antibodies have been obtained.

        Thus, this shuffling process revealed many more
heavy and light chain partners that bound to gp120
that were equal in affinity to those obtained from the

25   original library prepared in Example 2. With this
approach, additional HIV-1 neutralizing antibodies can
easily be obtained over those present in an original
library. The complexity of the clones arising from
the heavy chain shuffling also suggests that this

30   approach may be used to map the course of somatic
diversification.

        Combinatorial libraries randomly recombine heavy
and light chains so to what extent antibodies derived
from such libraries represent those produced in a

35   response _in vivo_ can be determined. In principle, a

heavy-light chain combination binding antigen could arise fortuitously, i.e., neither chain is involved in binding antigen _in vivo_ but the combination does bind antigen _in vitro_.

5   The available data suggests, however, that heavy chains, from immune libraries, involved in binding antigen tightly _in vitro_ arise from antigen-specific clones _in vivo_. First, studies have generally failed to identify high-affinity binders in non-immunized IgG

10   libraries. See, Persson et al. _Proc. Natl. Acad. Sci., USA_, 88:2432-2436 (1991) and Marks et al. _Eur. J. Immunol._, 21:985-991 (1991).

      Further, as described above, gp120 binders were not observed in panning a bone marrow IgG library from an HIV seronegative donor against gp120. Second,

15   heavy chains associated with binders from immunized libraries were typically at relatively high frequency in the library indicating they were strongly represented in the mRNA isolated from immunized

20   animals. See, Caton et al., _Proc. Natl. Acad. Sci., USA_, 87:6450-6454 (1990) and Persson et al., _supra_. Third, heavy chains from immunized libraries appeared to dictate specificity when recombined with various unrelated light chains as described in Example 10.

25   Fourth, the isolation of intraclonal heavy chain variants as here indicated that an active antibody response was cloned. Thus, the shuffling of a known heavy chain with a light chain binder and vice versa is preferred for use in this invention as new

30   neutralizing Fabs can be obtained beyond those generated _in vivo_.

      Heavy chain promiscuity, i.e., the ability of a heavy chain to pair with different light chains with retention of antigen affinity, presents serious

35   problems for identifying _in vivo_ light chain partners.

This applies not only to the strict definition of
partners as having arisen from the same B-cell but
also to one which would encompass somatic variants of
either partner.   The existence of predominant
heavy-light chain combinations, particularly involving
intraclonal light chain variants, suggests that the
light chains concerned are well represented in the
library and probably are associated with antigen
binding in vivo.   However, promiscuity means that,
although some combinations probably do occur in vivo,
one cannot be certain that one is not shuffling immune
partner chains in the recombination.   For instance,
the occurrence of a virtually identical light chain
(b6, B20) in 2 out of 33 clones suggests that it is
probably over-represented in the library consistent
with an in vivo involvement in antigen-stimulated
clones.   However, there is no way of knowing whether
the in vivo partner of the light chain is the b6 or
B20 heavy chain or indeed another heavy chain arising
from a stimulated clone.

The light chains arising from the combinatorial
library may not be those employed in vivo.
Nevertheless it is interesting to note that some heavy
chains appear relatively choosy about light chain
partner whereas others appear almost indifferent.
This observation needs to be tempered by the finding
that apparently choosy heavy chains from this analysis
will accept diverse light chains with maintenance of
antigen binding in a binary plasmid system where
pairings are forced as shown below in Example 11
rather than selected in a competitive situation.

Two reports compare heavy-light chain
combinations arising from combinatorial libraries and
hybridomas in immunized mice.   The library approach
begins with mRNA and is therefore probably reflecting

plasma cell populations. In contrast, hybridomas are thought to reflect activated but not terminally differentiated B cell populations and EBV transformation to reflect resting B cell populations.

5   Whatever the arguments about light chain authenticity, the heavy chains of Figure 10 present many features of interest. The most frequently used heavy chain is of the clone b8 type. It could be argued that this usage simply represents bias in PCR

10   amplification. However, the occurrence of approximately equal numbers of clones in this group amplified by VH1a and VH3a primers argues against this notion. Furthermore, the existence of intraclonal variants in some groups indicates that one is at least

15   sampling different genes from the initial library.

The antibodies cloned here do bear qualitative relationship with the polyclonal antibodies present in the serum of the asymptomatic donor. The titer of anti-gp120 (IIIB) antibodies was approximately 1:3000,

20   with greater than 50% of the reactivity being inhibited by CD4 or a cocktail of Fabs from clones 12, 13 and 14. The titer of anti-gp120 (SF2) antibodies was approximately 1:800. Further, the titer of serum against the short constrained V3 loop peptide was

25   1:500 and against the full length MN V3 loop peptide was only 1:300. The importance of "anti-CD4 site antibodies" seems general in donors with longer term HIV infection in that the cocktail of Fabs 12, 13 and 14 was able to inhibit binding of a large fraction of

30   serum antibody reactivity with gp120 (IIIB) in 26 of 28 donors tested.

The ability of Fabs to neutralize viruses has been a controversial area. One of the problems has been that Fabs are classically generated by papain

35   digestion of IgG. If the Fab, as is often the case,

shows reduced activity relative to the parent IgG then it may be difficult to rule out IgG contamination in the Fab preparation. Recombinant Fabs, however, as shown herein definitively neutralize virus.

5   The mechanism of neutralization of HIV-1 appears to neither require virion aggregation nor gp120 cross-linking. In addition, there is no correlation with blocking of the CD4-gp120 interaction to neutralization. The existence of the cloned

10 neutralizing Fabs of this invention should allow the molecular features that confer neutralizing potential to be explored. For instance, in the case of the group of clones containing Fab 13, the unique character of the light chain of that neutralizing

15 clone suggests that chain shuffling experiments in which the 13 light chain was recombined with the other heavy chains in that group, might be revealing. Heavy chains paired with two dissimilar light chains have been shown to retain antigen affinity but exhibit

20 altered fine specificity as shown in Example 11.
    The observation here of a large number of Fabs with only a limited number being strongly neutralizing may have important consequences. If the pattern is repeated for whole antibodies then it would seem that

25 much of the gp120 structure may be in a sense a "decoy", i.e., the immune system may invest considerable effort in producing antibodies of high affinity but limited anti-viral function. To exacerbate the situation the ineffective antibodies

30 may bind to gp120 and inhibit the binding of strongly neutralizing antibodies. This has obvious consequences for vaccination which should be primarily designed to elicit neutralizing antibodies of this invention.

35

10. Shuffling of Selected Heavy and Light Chain DNA
     Sequences of a Combinatorial Library in a Binary
     Plasmid System

     A binary system of replicon-compatible plasmids
has been developed to test the potential for
promiscuous recombination of heavy and light chains
within sets of human Fab fragments isolated from
combinatorial antibody libraries. The efficiency of
the system is demonstrated for the combinatorial
library of this invention derived from the bone marrow
library of an asymptomatic HIV donor.

     a.   Construction of the Binary Plasmid System
          The binary plasmids pTAC01H and pTC01 for
use in this invention contain the pelB leader region
and multiple cloning sites from Lambda Hc2 and Lambda
Lc3, respectively, and the set of replicon-compatible
expression vectors pFL281 and pFL261. Both pFL281 and
pFL261 have been described by Larimer et al., Prot.
Eng., 3:227-231 (1990), the disclosure of which is
hereby incorporated by reference. The nucleotide
sequences of pFL261 and pFL281 are in the EMBL,
GenBank and DDBJ Nucleotide Sequence Databases under
the accession numbers M29363 and M68946. The plasmid
pFL281 is based on the plasmid pFL260 also described
by Larimer et al., supra, and having the accession
number M29362. The only distinction between the
plasmids pFL260 and pFl281 is that pFL281 lacks a 60
bp sequence of pFL260 between the Eag I site and the
Xma III site resulting in the loss of one of the two
BamH I sites. This deletion is necessary to allow for
cloning of the BamH I Hc2 fragment into the expression
vector as described herein.

     The replicon-compatible expression vectors share
three common elements: (i) the f1 single-stranded DNA

page intergenic IG regions; (ii) the tightly regulated
tac promoter and lac operator; and (iii) an rbs-ATG
region with specific cloning sites. The plasmid
vectors differ in their antibiotic resistance markers

5   and plasmid replicons: pFL261 carries a gene encoding
chloramphenicol acetyltransferase (cat), conferring
chloramphenicol resistance,a nd the p15A replicon;
pFL281 carries a gene encoding beta-lactamase (bla),
conferring ampicillin resistance, and the ColE1

10   replicon (ori) from pMB1. The p15A and ColE1
replicons permit the coincident maintenance of both
plasmids in the same E. coli host.

The Hc2 and Lc2 vectors prepared in Examples 1a2)
and 1a3), respectively, were converted into the

15   plasmid form using standard methods familiar to one of
ordinary skill in the art and as described by Sambrook
et al., Molecular Cloning: A Laboratory Manual, 2nd
ed., Cold Spring Harbor Laboratory Press, New York
(1989) and subsequently digested with Xho I-Spe I

20   (pHc2) and Sac I-Xba I for (pLc2). The synthetic
linkers for insertion into the digested pHc2 and Lc2
plasmids were prepared by American Synthesis. The
linkers were inserted to increase the distance between
cloning sites so as to increase the effectiveness of

25   the digestions. The 5′ and 3′ linkers for preparing
the double-stranded linker insert into pHc2 were 5′
TCGAGGGTCGGTCGGTCTCTAGACGGTCGGTCGGTCA 3′ (SEQ ID NO
133) and 5′ CTAGTGACCGACCGACCGTCTAGAGACCGACCGACCC 3′
(SEQ ID NO 134), respectively. The 5′ and 3′ linkers

30   for preparing the double-stranded linker insert into
pLc2 were 5′ CGGTCGGTCGGTCCTCGAGGGTCGGTCGGTCT 3′ (SEQ
ID NO 135) and 5′
CTAGAGACCGACCGACCCTCGAGGACCGACCGACCGAGCT 3′ (SEQ ID NO
136), respectively. The pairs of linker

35   oligonucleotides were separately ligated to their

respective digested, calf intestinal phosphatase-
treated vectors.

Subsequently, the multiple cloning sites of pHc2
and pLc2 were transferred into the expression vectors,
pFL281 and pFL261, respectively. To accomplish this
process, the multiple cloning regions of both Lc2 and
Hc2 were separately amplified by PCR as described by
Gram et al., <u>Proc. Natl. Acad. Sci., USA</u>, 89:3576-3580
(1992) and as described in Example 2b using Vent
Polymerase (New England Biolabs) according to the
manufacturer's recommendations. The forward primer,
5' CAAGGAGACA<u>GGATCC</u>ATGAAATAC 3' (SEQ ID NO 137) was
designed to provide a flush fusion of the pelB leader
sequence to the ribosome binding sites of the cloning
vectors pFL261 and pFL281 via its internal BamH I site
indicated by the underlined nucleotides. The reverse
primer 5' AGGGCGAATT<u>GGATCC</u>CGGGCCCCC 3' (SEQ ID NO 138)
was designed to anneal downstream of the region of
interest in the parent vector of pHc2/pLc2 and create
a second BamH I site. The resultant Hc2 and Lc2 PCR
amplification products were then digested with BamH I
to provide for BamH I overhangs for subsequent
ligation into BamH I linearized pFL281 and pFL261
vectors, respectively. The resulting light chain
vector containing the Lc2 insert, designated pTC01,
was used in this form, whereas the heavy chain vector
was further modified with a histidine tail to allow
purification of Fab fragments by immobilized metal
affinity chromatography as described by Skerra et al.,
<u>Bio/Technology</u>, 9:273-278 (1991). For this purpose,
the synthetic linker oligonucleotides, respectively
the 5' and 3' linkers, 5'
CTAGTCATCATCATCATCATTAAGCTAGC 3' (SEQ ID NO 139) and
5' CTAGGCTAGCTTAATGATGATGATGATGA '3 (SEQ ID NO 140)
was inserted into the Spe I site, in effect removing

the decapeptide tag sequence to generate the heavy
chain vector designated as pTAC01H. The expression of
Fab fragment in all subsequent cloning experiments was
suppressed by adding 1% (w/v) glucose to all media and
5   plates.

        b.   Construction of Expression Plasmids
            For expression of the light chain variable
domain, pTC01 prepared above was first digested with
10   Sac I and Xba I; individual light chain inserts were
then obtained by separately digesting 22 of the
pComb2-3 plasmids prepared and screened as described
in Example 2 and listed in Figure 7 that bind to gp120
with the same combination of enzymes and isolating the
15   0.7 kb fragment using low melting point agarose gel
electrophoresis followed by b-agarose digestion. For
the chain-shuffling experiments, the following
representative members of each of the seven groups
shown in Figure 7 were chosen: b11; b6; b4-b12-b7-b21;
20   b3; s8; b1-b14-b24; b13-b22-B26-b8-b18-b27-B8-B35-s4;
and one loop peptide-binding clone, p35. The
different groups are indicated by semicolon
separations while members of the same group are
dashed. The resultant isolated light chains were
25   separately ligated into PTC01 overnight at 16°C under
standard conditions using a 5:1 molar insert-to-vector
ratio to form 21 light chain pTC01 expression vectors.
For expression of the heavy chain variable domain,
pTAC01H prepared above was first digested with Xho I
30   and Spe I; heavy chain inserts were then obtained by
separately PCR amplification reactions of the 20
pComb2-3 plasmids from which light chain inserts were
obtained. PCR was used to isolate the heavy chain
inserts instead of restriction digestion in order to
35   obtain heavy chain without the cpIII gene anchor

sequence in the vector. For the PCR reaction, the
respective 5' and 3' primers, 5'
CAGGTGCAGCTCGAGCAGTCTGGG 3' (VH1a) (SEQ ID NO 42) and
5' GCATGTACTAGTTTTGTCACAAGATTTGGG 3' (CG1z) (SEQ ID NO

5   44) were used to amplify the region corresponding to
the heavy chain as described in Examples 2a1) and
2a2). The resultant PCR products were purified by
low-melting point electrophoresis, digested with Xho I
and Spe I, re-purified, and separately ligated to the

10   similarly prepared heavy chain pTAC01H vector using a
1:2 molar vector-to-insert ratio to form 21 heavy
chain pTAC01H expression vectors.

     c.   Co-transformation of Binary Plasmids

15   CaCl$_2$-competent XL1-Blue cells (Stratagene;
recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, lac,
{F' proAB, lacI^q, ZDM15, Tn10(tet^R)}) were prepared
and transformed with approximately 0.5 μg purified DNA
of each plasmid in directed crosses of each of the 20

20   light chain vectors with each of the 20 heavy chain
vectors. The presence of both plasmids and the
episome was selected for by plating transformants on
triple-antibiotic agar plates (100 μg/ml
carbenicillin, 30 μg/ml chloramphenicol, 10 μg/ml

25   tetracycline, 32 g/l LB agar) containing 1% glucose.
     A binary plasmid system consisting of two
replicon-compatible plasmids was constructed as shown
in 14. The pTAC01H heavy chain vector schematic is
shown in Figure 14A and the pTC01 light chain vector

30   schematic is shown in Figure 14B. Both expression
vectors feature similar cloning sites including pel B
leader sequences fused to the ribosome binding sites
and the tac promoters via BamH I sites as shown in
Figure 15. The nucleotide sequences of the multiple

35   cloning sites along with the tac promoter, ribosome

binding sites (rbs) and the underlined relevant restriction sites for the light chain vector, pTCO1, and heavy chain vector, pTAC01H, are respectively shown in Figure 15A and Figure 15B. The sequences are also listed in the Sequence Listing as described in the Brief Description of the Drawings. The heavy chain vector pTAC01H also contains a $(His)_5$-tail to allow purification of the recombinant Fab fragments by immobilized metal affinity chromatography. The presence of both plasmids in the same bacterial cell is selected for by the presence of both antibiotics in the media. Expression is partially suppressed during growth by addition of glucose and induced by the addition of IPTG at room temperature. Under these conditions, both plasmids are stable within the cell and support expression of the Fab fragment as assayed by ELISA using goat anti-human kappa and goat anti-human IgG1 antibodies.

d.   Preparation of Recombinant Fab Fragments
Bacterial cultures for determination of antigen-binding activity were grown in 96 well-tissue culture plates (Costar #3596). 250 μl Superbroth [SB had the following ingredients per liter: 10 g 3-(N-morpholino) propanesulfonic acid, 30 g tryptone, 20 g yeast extract at pH 7.0 at 25°C) containing 30 μg/ml chloramphenicol, 100 μg/ml carbenicillin, and 1% (w/v)] glucose were admixed per well and inoculated with a single double-transformant prepared in Example 11c above. The inoculated plates were then maintained with moderate shaking (200 rpm) on a horizontal shaker for 7-9 hours at 37°C, until the A_{550} was approximately 1-1.5. The cells were collected by centrifugation of the microtiter plate (1,500 X g for 30 minutes at 4°C), the supernatants were discarded, and the cells

were resuspended and induced overnight at room
temperature in fresh media containing 1 mM IPTG, but
no glucose. Cells were harvested by centrifugation,
resuspended in 175 μl PBS (10 mM sodium phosphate, 160

5   mM NaCl at pH 7.4 at 25°C) containing 34 μg/ml
phenylmethylsulfonyl fluoride (PMSF) and 1.5% (w/v)
streptomycin sulfate, and lysed by 3 freeze-thaw
cycles between -80°C and 37°C. The resultant crude
extracts were partially cleared by centrifugation as

10   above before analysis by antigen-binding ELISA.

          e.   Assay and Determination of Relative
                Affinities

15   Relative affinities were determined as
described in Example 2b6) after coating wells with 0.1
μg of antigen. The selected antigens included tetanus
toxoid and recombinant gp120 (strain IIIB) and gp120
(strain SF2). For each antigen, a negative control

20   extract of XL1-Blue cells co-transformed with pTC01
and pTAC01H was tested to determine whether other
components in E. coli had any affinity for the
antigens in the assay. Each extract was assayed for
BSA-binding activity and BSA-positive clones were

25   considered negative. All possible single-
transformants expressing one chain only were prepared
as described for the double-transformants and were
found to have no affinity for any of the antigens
used. Because of the nature of the assay, whether

30   this was due to a lack of binding by the individual
chains itself or due to a lack of expression or
folding could not be determined.

          f.   Results of Direct Crosses of Heavy and
35   Light Chains within a Set of gp120/gp160

Binding Antibodies

The Fab fragments derived from the bone
marrow of the same asymptomatic HIV donor but panned
against gp120 (IIIB), gp160 (IIIB), and gp120 (SF2),
were assigned to one of seven groups based on the
amino acid sequences of the CDR3 of their heavy chains
as described in Example 9. From the same library,
antibodies to the constrained hypervariable v3-loop-
like peptide JSISIGPGRAFYTGZC (SEQ ID NO 141) were
isolated. For the chain-shuffling experiments, the
following representative members of each of the seven
groups shown in Figure 7 were chosen: b11; b6; b4-b12-
b7-b21; b3; s8; b1-b14-b24; b13-b22-B26-b8-b18-b27-B8-
B35-s4; and one loop peptide-binding clone, p35.
Clones b4, b7, b12, and b21 showed neutralization
activity against HIV when monitoring inhibition of
infection by syncytia formation and clones b13, b12,
and b4 when monitoring p24 production as shown in
Example 3. Light and heavy chains were cloned from
the original constructs and cotransformed in all
possible binary combinations into XL1-Blue cells as
described above.

The results of the complete cross are shown in
Figure 16. As is to be expected, identical chains
derived from different Fab fragments had similar
binding properties e.g., b18HC, b27HC, B8HC, B35HC,
s4HC. The crosses of the original heavy chains with
the original light chains in each case clearly
recapitulated binding activity. Minor differences
existed between some heavy chains with identical
variable domain sequences, e.g., b4 and b12 (constant
domains were not sequenced for any of the constructs).
The exception is b8HC, which was identical in its
variable domain to b18HC, b27HC, B8HC, B35HC, s4HC,
yet shows more cross reactivity. Presumably, this is

due to differences in expression levels in the cell or
differences in the constant domain sequences. Clear
differences existed between heavy chains in their
tendency to accept different light chains and still

5   bind antigen, but even the least promiscuous heavy
chain in the set panned against gp120 (IIIB), b1HC,
still did so in 43% of its crosses. On the other side
of the spectrum, 5 heavy chains, b11HC, b6HC, b12HC,
b7HC, and b8HC, crossed productively with all light

10   chains in this set. For the heavy chain crosses
examined in detail (all of s4HC, B35HC, B26HC; most of
b12HC, b12HC), no significant differences in apparent
binding affinity were found between Fab fragments
using the same heavy chain but different light chains

15   as shown in Figure 17 where the IC_{50} from competition
with soluble gp120 (IIIB) was approximately 10^{-8} M.

Within the original seven groups that were
established according to the sequence of the CDR3 of
the heavy chains and that are indicated by horizontal

20   and vertical lines in Figure 16, complete promiscuity
was present, i.e., heavy and light chains within these
CDR3-determined groups were completely promiscuous
with each other. However, there was a lack of
promiscuity between other groups, e.g., between

25   b1HC-b24HC and b13LC-s4LC. In the analysis of these
sequence-based groups, the protein antigen against
which the phage display library was panned was not a
critical factor. The exception to this case was the
cross of p35HC with all light chains; the only cross

30   that bound either to gp120 (SF2 strain) or the
original antigen, the loop peptide, was the cross
containing the original heavy and light chains.

Unlike the heavy chains, no light chains crossed
productively with all heavy chains nor were any

35   distinguishable from the other light chains by

unusually low promiscuity.

In the neutralization assays performed as
described in Example 3, the directed cross resulting
from the pairing of the heavy chain from clone b12
with the light chain from clone b21, was effective at
neutralizing HIV-1.

g.   Interantigenic Crosses of Heavy and Light
      Chains

To determine whether conclusions derived
from the crosses between high affinity Fab fragments
originating from the same library can be extended to
unrelated libraries, a non-related gamma1k-Fab
fragment (P3-13) specific for tetanus toxoid from a
different donor was chosen for a new set of crosses
[clone 3 in Persson et al., Proc. Natl. Acad. Sci.,
USA, 88:2432-2436 (1991)]. Extracts were probed with
tetanus toxoid or with gp120 (IIIB). The data confirm
the results from the gp120 cross experiment in that
the binding activity towards the antigen was
determined by the heavy chain. The heavy chain of
clone P3-13 paired with the light chains b4, b12, b21,
and b14 to yield an Fab fragment with an affinity
towards tetanus toxoid; the light chain of P3-13
paired with the heavy chains of b3, b6, b11, and b14
to yield an Fab fragment with an affinity towards
gp120 (IIIB). None of the light chains originating
from the gp120 binders was able to confer gp120
specificity in combination with the P3-13 heavy chain.
Similarly, the P3-13 light chain was unable to
generate tetanus toxoid specificity in combination
with any of the heavy chains originating from the
gp120 binders, confirming the dominance of the heavy
chain in the antibody-antigen interaction.
Interestingly, all three light chains that showed a

strong signal against tetanus toxoid (b4, b12, b21)
were members of the same group when sorted by the
CDR3's of their original heavy chains. As might be
expected from crosses between unrelated libraries, not

5   only was there a lower degree of promiscuity, i.e.,
chains paired productively with far fewer
complementary chains, but the range of apparent
affinity constants determined by competition ELISA was
much broader (6.3 X 10^6 - 6.3 X 10^{-8} M). The

10   replacement of the original P3-13 light chain in the
P3-13 Fab fragment with another light chain lowered
the affinity of the Fab towards tetanus toxoid 10 to
100-fold (from 6.3 X 10^{-8} M to 6.3 X 10^{-6} M). In the
crosses of the light chain of P3-13 with all the heavy

15   chains of the HIV pannings, the productive crosses had
similar affinities to gp120 (IIIB) (2.5 X 10^7 - 6.3 X
10^{-7} M), with the exception of b14HC/P3-13LC, whose
signal was too weak for a definite determination of
the apparent binding constant. These affinities were

20   approximately five-fold lower than those of the
gp120-heavy chains with their original light chains.
          Thus, the results show that chain shuffling is
yet another maneuver allowed _in vitro_ but not _in vivo_
which can be expected to help extend antibody

25   diversity beyond that of Nature. The overriding
feature of the binary system of this invention is its
ability to create large numbers (several hundred) of
directed crosses between characterized light and heavy
chains without the need for recloning individual

30   chains for each cross after the initial vector
construction. When used in combination with the
phage-display method and biological assays, it allows
the rapid analysis of the most interesting subset of
the pool of antigen-binding clones by chain shuffling,

35   with the aim of finding biologically or chemically

active antibodies. For the set of antigens studied here, most heavy chains recombined with a number of light chains to yield an antigen-binding Fab fragment.

These results have important implications for the diversity of combinatorial antibody libraries. While it is not possible to predict reliably the original in vivo combinations of light and heavy chains due to the surprising promiscuity of individual chains, recombinant antibody libraries take advantage of the fact that even distantly related Fabs against the same antigen can recombine in vitro to give chain combinations not found in vivo. In fact, after the identification of a certain number of antibodies that have been shown to possess some biological or chemical activity, it may be better to shuffle their individual chains in a directed fashion than to continue sampling randomly from the same pool of binders. By extension, the promiscuity observed in this system indicates that in libraries constructed using degenerate, chemically synthesized oligonucleotides, there should be considerable flexibility in which separate synthetic heavy chains can pair with separate synthetic light chains to generate separate antigen-binding Fab fragments. The diversity of combinatorial libraries coupled with chain-shuffling should allow wide exploration of three dimensional space thereby solving the problem of how to approximate molecules in the ternary complex of antibody, substrate and cofactor.

11. Deposit of Materials

The following cell lines have been deposited on September 30, 1992, with the American Type Culture Collection (ATCC), 1301 Parklawn Drive, Rockville, MD, USA:

Cell Line		ATCC Accession No.
E. coli	MT11	ATCC 69078
E. coli	MT12	ATCC 69079
E. coli	MT13	ATCC 69080

The deposits listed above, MT11, MT12 and MT13 are bacterial cells (_E. coli_) containing the expression vector pComb2-3 for the respective expression of the Fabs designated b11 (clone b11), b12 (clone b12), and b13 (clone b13) prepared in Example 2b. The sequences of the heavy and light chain variable domains are listed in Figure 10 and 11, respectively. This deposit was made with the ATCC under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from the date of deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty which assures permanent and unrestricted availability of the progeny of the culture to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 U.S.C. §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638). The assignee of the present application has agreed that if the culture deposit should die or be lost or destroyed when cultivated under suitable conditions, it will be promptly replaced on notification with a viable specimen of the same culture. Availability of

the deposited strain is not to be construed as a
license to practice the invention in contravention of
the rights granted under the authority of any
government in accordance with its patent laws.

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The foregoing written specification is considered
to be sufficient to enable one skilled in the art to
practice the invention. The present invention is not
to be limited in scope by the cell lines deposited,
10 since the deposited embodiment is intended as a single
illustration of one aspect of the invention and any
cell lines that are functionally equivalent are within
the scope of this invention. The deposit of material
does not constitute an admission that the written
15 description herein contained is inadequate to enable
the practice of any aspect of the invention, including
the best mode thereof, nor is it to be construed as
limiting the scope of the claims to the specific
illustration that it represents. Indeed, various
20 modifications of the invention in addition to those
shown and described herein will become apparent to
those skilled in the art from the foregoing
description and fall within the scope of the appended
claims.

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